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An Hypothesis Paper

Neural Networks vs Principal Component Regression for Prediction of Wheat Flour Loaf Volume in Baking Tests

Y. HORIMOTO, T. DURANCE, S. NAKAI, and O.M. LUKOW

ABSTRACT

Neural networks (NN) provide a simple means of predicting outcomes that depend upon complex, possibly nonlinear, relationships between many variables. A trained neural network was created and used to predict loaf volume of breads made from different wheat cultivars. Although creating the NN required specialized skills and considerable computational time, using the "trained" NN to estimate remix loaf volume, was very rapid and required only basic computer skills. Random Centroid Optimization (RCO) was also employed to choose the best training parameters: learning rate = 0.820, smoothing factor = 0.723, noise = 0.056, number of hidden neurons = 5. NN was more accurate, faster and easier than Principal Component Regression Analysis.

Key Words: neural network, principal component regression, wheat flour, loaf volume, bread

INTRODUCTION

ACCURATE laboratory assessment of the bread-making quality of wheat is essential for development of new cultivars that will meet commercial specifications for domestic and export markets. Acceptable bread-making quality is defined by narrow limits within a wide range of physical, chemical, and rheological properties of wheat. Many standardized tests for bread-making quality, particularly those involving baking, are time-consuming, costly and require large samples. One approach used by cereal chemists has been multiple linear regression analysis to identify samples with high bread-making potential based on a few rapid, small-scale flour tests (Lukow, 1991).

A technique known as neural networks may be applicable to predict baking quality based on standard flour quality tests.

A variety of multivariate methods have been used in food science and technology (Aishima and Nakai, 1991; Resurreccion, 1988). Statistical and chemometric techniques, such as regression, partial least squares and principal component regression analysis have provided satisfactory predictions (Aishima and Nakai, 1991). For example, multiple linear regression analysis identified wheat breeder's lines with potentially large loaf volume instead of using laborious time-consuming bake tests. For such techniques, however, proper application requires expertise and care. Also, preliminary assumptions must be made regarding expected nature and dimensionality of the relationships (Aishima and Nakai, 1991; Kowalski, 1984).

Artificial neural networks have become a focus of interest in many areas including food science. A neural network (NN) is a computer based, information processing technique which attempts to simulate the functions of living nervous systems (Eberhart and Dobbins, 1990). Biological organisms can simultaneously evaluate many different kinds of stimuli for problem solving. Recognizing a voice on the telephone provides an example. We incorporate factors such as tone, pitch, vocabulary, information content, emotional clues, etc. and give each factor weight depending upon past experience. This approach, called parallel information processing, is fundamentally different from sequential processing used, for example, to multiply two large numbers. Conventional computers are sequential processors but some software simulates parallel processing as is the case with most commercial NN software.

A NN is created or "trained" to solve a particular problem by examining a large number of samples in which both influencing factors (in our case the flour quality test results) and dependent yield variable (remix loaf volume) are known. In this sense, NN analysis is similar to multiple linear regression and related statistical techniques. NN is different in that the relationships between influencing factors and yield variables are estimated by an iterative, trial and error procedure. Different weights are applied to each influencing factor and the combined weighted factors are added to predict the dependent yield variable (in our case loaf volume). The procedure is repeated until a set of weights is found that can correctly predict yields of the training data set. The NN can then be used to predict unknown yields of new samples.

A major theoretical advantage of NN over statistical methods such as multiple linear regression or principle component regression is that NN does not impose a linear relationship between factors and yield. This is a result of the method used to convert inputs into outputs, especially the threshold value concept that can be incorporated. Artificial neurons add inputs, that is weighted measures of influencing factors, and create output, usually a 0 or 1 type response. Until the

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sum of inputs exceeds a threshold, output is zero. Typically the outputs of many neurons are combined so the total output may not be simply "yes" or "no" but outputs of individual neurons need not be linearly related to input. Nonlinear regression is much less developed than linear statistical methods and experimenters often are forced to assume linearity for such statistical predictions. If the data deviate far from that assumption, predictions will be ineffective and a NN approach may be more useful. If the relationship is linear, either approach should be satisfactory.

There are two principal types of network architecture: feed forward and feed back (Lawrence, 1991). The most popular feed forward method is back-propagation, and such NN are called back-propagation networks (BPN). BPN has been extensively studied, theoretically and experimentally and has been the most successful (Eberhart and Dobbins, 1990; Jansson, 1991; Lawrence, 1991; Wythoff, 1993).

The BPN is usually built from three layers (Fig. 1): input, hidden, and output. The first layer (input) takes the input values of a "pattern." In our case a pattern is a set of flour quality test measures for an individual wheat cultivar. The last layer (output) produces the pattern or sample output. The layers between are called the hidden layers. Each layer is made up of neurons. Input and output neurons are places to store or generate a number. The strength of a connection between two neurons is the weight. It determines the effect one neuron can have on another (Eberhart and Dobbins, 1991). The input is multiplied by its weight to give a signal between two neurons. Weighted signals are summed to provide a net value. Usually they are simply added. An equation in the hidden layers (transfer function) determines whether inputs are sufficient to produce an output. There are several kinds of transfer functions, e.g., threshold or sigmoid functions (Lawrence and Peterson, 1992). In training a NN, the values predicted by the network are compared to experimental values using the delta rule, an equation which minimizes errors between experimental values and network predicted values (Lawrence, 1991). The errors are then back-propagated to hidden and input layers to adjust weights. This is repeated many times until errors between predicted and experimental values are minimized. General reviews and references of NN procedures were published by Eberhart and Dobbins (1990), Jansson (1991), Lawrence (1991), and Wythoff (1993).

Trained neural networks qualify as intellectual property. They can be copyrighted and sold to potential users. Our objective was to use NN for prediction of a baking test of wheat flour dough. Principal component regression analysis (PCR) was also used for prediction and compared.

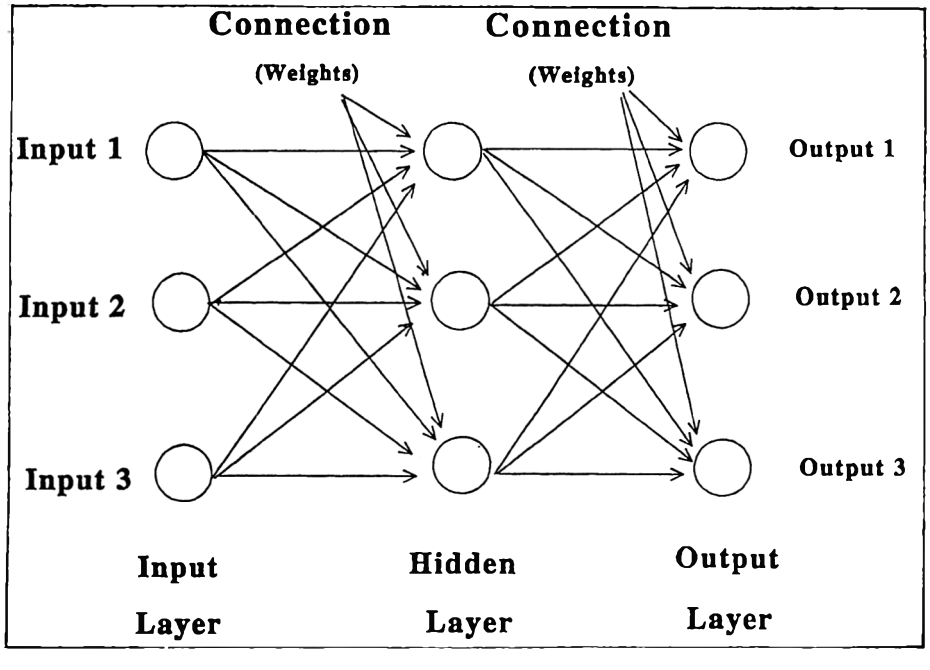

Fig. 1—Back-propagation network structure.

Table 1—Input variables for network

Input variables	
PRO:	% flour protein
FLY:	% flour yield
MDT:	mixograph development time
PKH:	Mixograph peak height
DDT:	far nograph dough development time
MTI:	far nograph mixing tolerance index
FAB:	% farinograph absorption
TEST:	test weight
MKW:	1000 kernel weight

MATERIALS & METHODS

Database

Quality tests of wheat cultivars (400 patterns) were performed at the Agriculture Canada Cereal quality Research Laboratory (Winnipeg, Manitoba). The quality tests (Table 1) were performed by methods of Lukow et al. (1990) and Lukow and McVeity (1991).

Network training procedures

A three-layer neural network was used to predict the baking test of wheat flour dough using the back-propagation algorithm. A sigmoid function was used as transfer function, because the sigmoid function is particularly useful for nonlinear relationships. Nine variables were used as "input neurons" for a network (Table 1). For each flour sample, one result or "output neuron" was predicted: remix loaf volume method (RLV). Training parameters for the network were selected by the random centroid optimization (RCO) technique (Nakai, 1990). The software program "Brainmaker" (California Scientific Software, CA) was used to build the NN.

Network optimization

Proper functioning of a neural network is highly dependent on the way signals are propagated through the network. The weights of connections are of great importance for this propagation and proper setting of weight factors is essential. In general, settings are not known beforehand and initially weights are artibrarily chosen. The choice of parameters for a network is empirical and differs for varying topologies and pattern sets. We used a random centroid optimization (RCO) method (Nakai, 1990) to develop the best architecture for the network.

Random centroid optimization

Random centroid optimization was used for determining an optimum neural

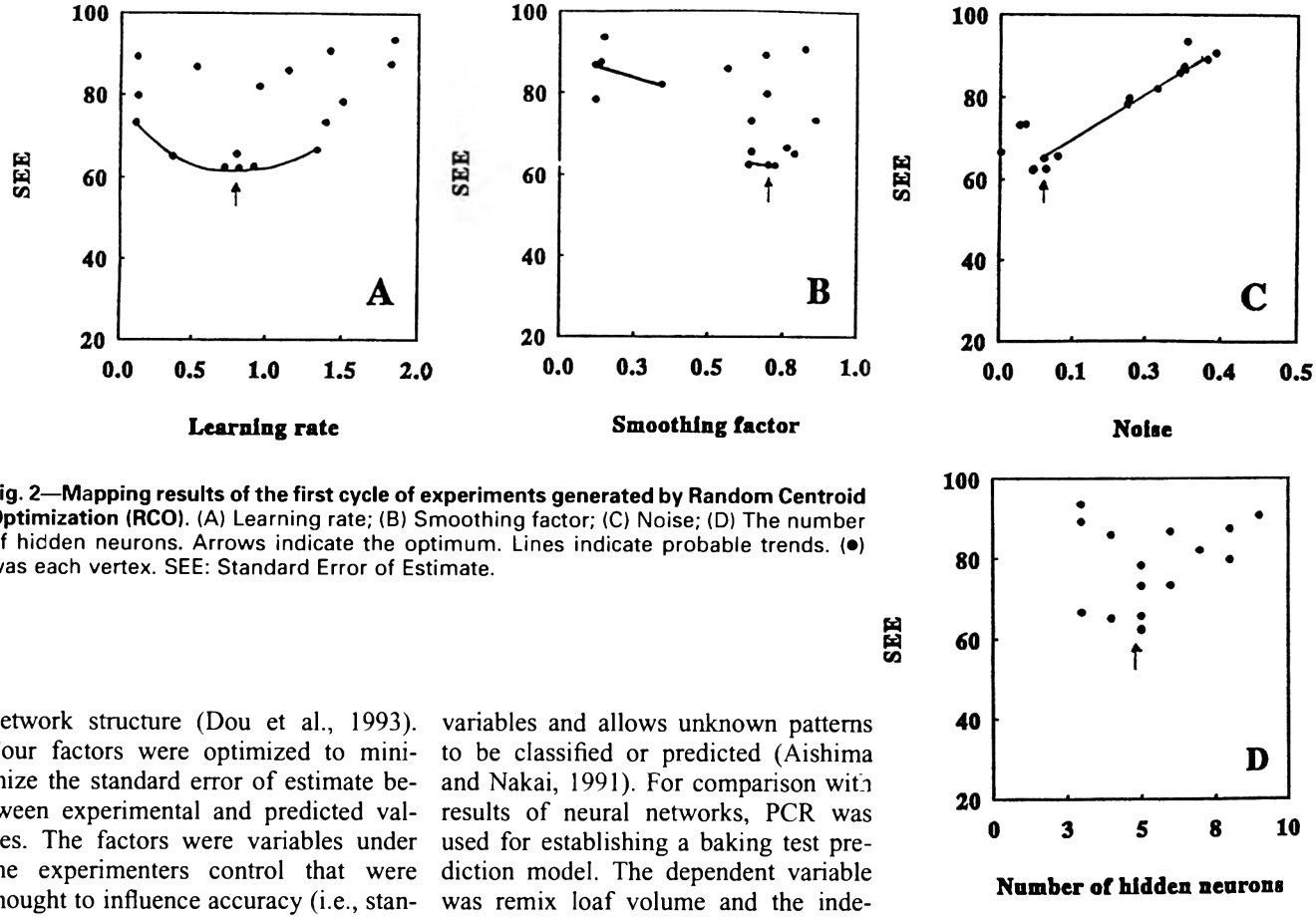

Fig. 2—Mapping results of the first cycle of experiments generated by Random Centroid Optimization (RCO). (A) Learning rate; (B) Smoothing factor; (C) Noise; (D) The number of hidden neurons. Arrows indicate the optimum. Lines indicate probable trends. (•) was each vertex. SEE: Standard Error of Estimate.

network structure (Dou et al., 1993). Four factors were optimized to minimize the standard error of estimate between experimental and predicted values. The factors were variables under the experimenters control that were thought to influence accuracy (i.e., standard error of estimate) of the NN: learning rate, smoothing factor, noise, and the number of hidden neurons. Based on reviews (Bos et al., 1992; Eberhart and Dobbins, 1990; Lawrence, 1991), the experimental ranges for each factor were established as follows: learning rate, 0.1-2.0; smoothing factor, 0.1-1.0; noise, 0-0.5; number of hidden neurons, 2-9.

Testing the network

After training, performance of the network was tested. Experimental RLVs were compared with RLVs predicted by the neural network. The disagreement between the two solutions gave an indication of performance of the trained network. Files established were a training file, and a testing file. Patterns (200) were used for a training file and different patterns (200) for a testing file. Squared multiple correlation (r^2) and standard error of estimate (SEE) were calculated between predicted and experimental values.

Principal component regression analysis (PCR)

PCR has been successfully used in food science, enables relating blocks of variables and allows unknown patterns to be classified or predicted (Aishima and Nakai, 1991). For comparison with results of neural networks, PCR was used for establishing a baking test prediction model. The dependent variable was remix loaf volume and the independent variables were the first five principal components.

Statistical procedure

All statistical analyses, including PCR and multiple correlation were performed using the statistical software SYSTAT (Wilkinson, 1990).

RESULTS & DISCUSSION

LEARNING RATE and momentum factors are important to the achievement of optimum neural networks (Bos et al., 1992; Eberhart and Dobbins, 1990; Lawrence, 1991). In normal back-propagation, the weight adjustments are multiplied by a learning rate before summing the weights. If the learning rate is low, the convergence of the weight set to an optimum is accurate, but very slow, and stalling in a local optimum may be a problem. If it is higher, the system may oscillate. Also, a momentum factor can be used to improve convergence. In the "Brainmaker" software, the smoothing factor is used to determine the momentum of the training algorithm. It determines how much of an error will be corrected at the time it is encountered, and how much will be averaged, or smoothed, into successive factors (Lawrence and Peterson, 1992).

It has been helpful to add noise to input and output patterns, especially if the data set contains irrelevant or imprecise data, in order to get a good prediction of NN (Lawrence and Peterson, 1992). Random values were added to all weights to prevent the network from stalling in a local minimum.

The number of hidden neurons is important for the effectiveness of a network. Network performance may vary with number of hidden neurons (Sigillito and Eberhart, 1990). With too many neurons, a network may not learn but memorize patterns, or it may train and run more slowly. On the other hand, without enough hidden neurons, a network may not become trained (Lawrence and Peterson, 1992).

Learning rate, smoothing factor, noise and hidden neuron number were optimized using Random Centroid Optimization (RCO) (Table 2). Values for parameters in each experiment were suggested by the RCO program, then used in NN experiments. The objective here was to determine values of four parameters which minimize the standard error of estimate (SEE) between prediction of NN and actual, measured remix

Table 2—Summary data for random-centroid optimization of neural networks

Vertex	Learning rate	Smoothing factor	Noise	Hidden neurons	SEE
1	0.528	0.120	0.316	6	86.65
2	1.144	0.566	0.307	4	85.83
3	0.901	0.912	0.230	2	229.09
4	0.122	0.697	0.221	8	79.71
5	0.954	0.345	0.269	7	82.01
6	1.392	0.860	0.043	6	73.36
7	0.109	0.645	0.034	5	73.21
8	1.416	0.825	0.366	9	90.66
9	1.841	0.150	0.319	3	93.49
10	1.506	0.122	0.218	5	78.24
11	1.819	0.139	0.314	8	87.40
12	0.115	0.696	0.352	3	89.17
13	1.333	0.763	0.002	3	66.60
14	0.365	0.789	0.076	4	65.14
15[a]	0.804	0.646	0.099	5	65.71
16[a]	0.918	0.637	0.079	5	62.53
17[b]	0.820	0.723	0.056	5	62.21
18[b]	0.725	0.701	0.058	5	62.40

[a]Centroid points
[b]Re-centroid points

Table 3—Error between known values and predicted values in training file[a]

Cultivars (n)	Iterations	Method	r^2	SEE
400	1,469	NN[b]	0.689	61.333
		PCR[c]	0.635	66.408

[a]Correlation coefficient (r^2) and standard error of estimate (SEE) were calculated using the predicted against the known value.
[b]Neural Network training procedure: Learning Rate: 0.820; Smoothing Factor: 0.723; Noise: 0.0560; Number of Hidden Neurons: 5; Training Tolerance (%): 10.
[c]Principal Component Regression Analysis.

Table 4—Correlation between known values and prediction values[a]

Iterations	Method	r^2	SEE
12,407	NN[b]	0.619	67.503
	PCR[c]	0.592	69.984
52,135	NN[b]	0.646	64.834
	PCR[c]	0.592	69.985

[abc]Footnotes same as Table 3

Table 5—Performance of neural networks by changing tolerance (N = 200)

Tolerance (%)	Testing file r^2	Testing file SEE
10	0.646	64.834
20	0.632	70.312
30	0.622	68.469

loaf volumes of the flour. Two hundred patterns (i.e., quality test results of 200 flours) were used for training the NN. Training was stopped after 1 hr (about 1000 iterations) and SEE was calculated between experimental values and predicted values. All data were mapped (Fig. 2A-2D). The mapping process aids in visualization of the experimental response surface, indicating the trend of the data (Nakai et al., 1984). RCO is usually repeated until one gets a response considered adequate. After the first cycle of the RCO program, the approximate position of the optimum was clear. The best result was obtained with learning rate, 0.820; smoothing factor, 0.723; noise, 0.056; and number of hidden neurons, 5 (Table 2). SEE was 62.217. A NN was trained with 400 patterns using the optimized parameters and the result compared favorably with PCR (Table 3).

The 400 cultivars were arbitrarily divided into two groups, a training file (N=200) and a testing file (N=200), and a new NN was trained with the optimized learning rate, smoothing factor, etc. After both 12,407 and 52,135 iterations, the NN training was stopped and performance was checked with the testing file and compared to PCR. Again, NN was better than PCR (Table 4). An NN with more iterations could predict better than one with less iterations. Correlations were compared between experimental values and predicted values in the testing file with 12,407 (Fig. 3) and 52,135 (Fig. 4) training iterations. For both figures, the NN results scattered more closely around the perfect 45° line than those of PCR analysis. However, even after 52,135 iterations, the network system was not completely optimized. This suggests that insufficient information was contained in the quality evaluation tests (Table 1) to predict Remix Loaf Volume with acceptable accuracy. For useful prediction, r^2 should be >0.9.

One criticism of NN is that it is not possible to determine how the network arrives at a particular conclusion: that is, the trained network is something of a "black box," the contents of which are hidden from the users (Collins, 1993). Thus the user may be uncertain if a particular NN is close to optimum or if they should continue searching. One possible method for testing the suitability of a trained network is to change the tolerance. The training tolerance specifies how close each output (i.e. remix loaf volume) of the network must be to the empirical response to be considered "correct" during training. The training tolerance is a percentage of the range of the output neuron (Lawrence and Peterson, 1992). Networks with smaller tolerances require longer to train. If a network is slow in learning, it is sometimes helpful to begin with a wide tolerance, and then narrow tolerance. In this case, the NN finished training with 20% tolerance (Table 5). After changing the tolerance to 30%, it took less time to finish training. SEE was better for this tolerance than in the 20% case. This was opposite of what we expected. Probably, there was not much difference in performance between 20 and 30% tolerance. When possible, it is best to train a network many times with different data sets but additional data sets of sufficient size were not available. The RCO results (Fig. 2) indicated that the chosen values for learning rate, smoothing factor, noise and number of hidden neurons were close to optimum. Based on these results and other reports (Lukow, 1991; Lukow et al., 1990; Lukow and McVeity, 1991) we concluded that the flour quality attributes available to us did not relate sufficient information to make a more accurate prediction. More or different quality parameters may be required. Also, 200 cultivars seems a large training set but we know of no method of estimating how large a training file may be needed for a particular NN. NN did consistently make slightly better predictions than Principal Component Regression Analysis, which until now has been the most successful multivariate technique for such use. Although much more theoretical and experimental work is needed, this technique can be employed as a simple, rapid computer technique for accurate prediction of loaf volume. Potential exists to apply this technique to quality screening programs of new cultivars, especially when insufficient volume of flour is available for baking tests. The NN approach is potentially more useful than other multivariate analysis methods such as PCR. which may be difficult for

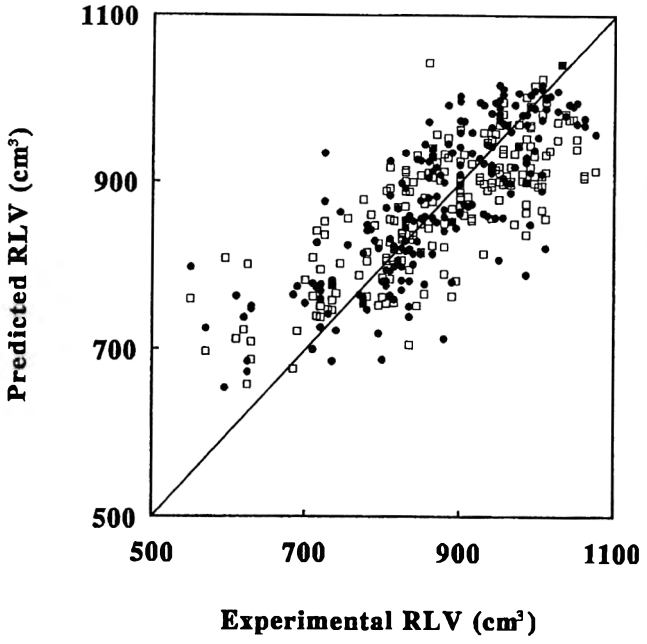

Fig. 3—Correlation of experimental remix loaf volumes (RLV) with RLV predicted by Neural Networks (NN-1) with 12,407 iterations (•) and Principal Component Regression (PCR) analysis (□). Solid line represents perfect correlation.

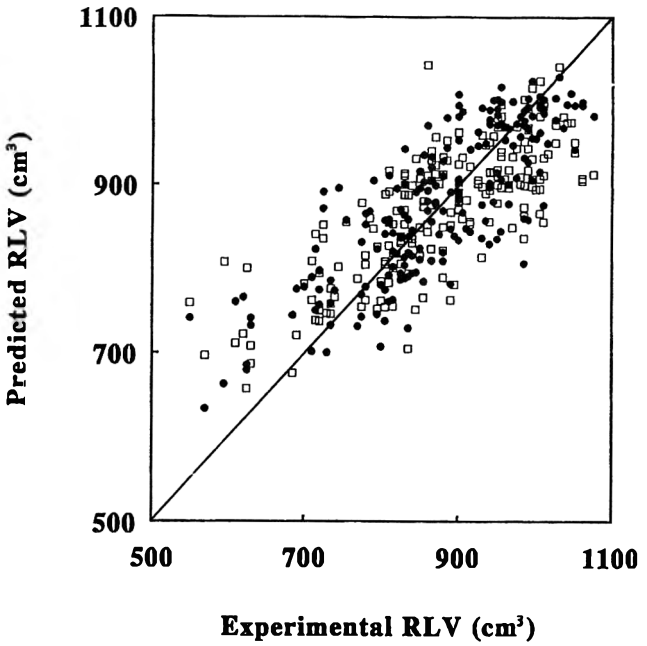

Fig. 4—Correlation of experimental remix loaf volumes (RLV) with RLV predicted by Neural Networks (NN-2) with 52,135 iterations (•) and Principal Component Regression (PCR) analysis (□). Solid line represents perfect correlation.

the untrained to carry out and understand. Use of a pretrained NN does not require a skilled statistician and output is unambiguous.

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Wheat Flour Compound that Produces Sticky Dough: Isolation and Identification

W.Z. CHEN and R.C. HOSENEY

ABSTRACT

Some wheats have a 1B/1R translocation and may produce flours that give sticky doughs, even with optimum mixing time and water absorption. Studies showed that the water-soluble fraction caused sticky dough. Dialysis experiments indicated that the responsible substance had low molecular weight. Ionic exchange showed it to be either neutral or negatively charged. Gel filtration chromatography indicated it was both carbohydrate and UV-absorbing. Saponification caused it to lose ability to cause sticky dough. The UV-absorbing material and the carbohydrate had to be linked for the compound to be active. HPLC mass spectrometry indicated the UV-absorbing fraction was ferulic acid and the carbohydrate was a hexose.

Key Words: wheat; flour; stickiness; ferulic acid

INTRODUCTION

WHEN WHEAT FLOUR AND WATER are mixed in correct proportions for optimum time, a smooth, homogeneous dough is formed (Hoseney and Finney, 1974). However, if a dough is overmixed or contains too much water, it can become extremely sticky (Hlynka, 1970; Hoseney and Finney, 1974). In a laboratory, sticky doughs are troublesome and must be handled carefully. However, in a large commercial bakery where hundreds or thousands of doughs may be produced in short times a sticky dough can cause major problems, even stopping production lines.

Sticky-dough problems caused by overmixing or excess water can be overcome by careful adjustment of those factors. However, the use of 1B/1R translocation in wheat breeding program introduced factors causing dough stickiness which cannot be controlled in the bakery (Law and Payne, 1983; Moonen and Zeven, 1984; Martin and Stewart, 1986).

Wheat breeding programs in many countries use 1B/1R translocated wheats. In 1B/1R wheats the short branch of chromosome 1B of wheat has been replaced by the short branch of chromosome 1R of rye. The latter chromosome carries genes that impart resistance to several important wheat diseases, viz. powdery mildew, stripe rust, leaf rust, and stem rust (Mettin et al, 1973; Zeller, 1973; Moonen and Zeven, 1984). Further, the fact that many high-yielding wheat cultivars have this translocation suggests that it may increase yield. Consequently, it is being used widely in breeding programs for both winter and spring wheats (Mettin and Bluthner, 1984; CIMMYT, 1985).

However, associated with those advantages are adverse effects of the 1B/1R translocation. Flours from such wheats reportedly produce doughs with marked stickiness, reduced strength, and intolerance to overmixing (Martin and Stewart, 1986). Our objectives were to isolate and identify the substance(s) that cause the sticky dough effects.

MATERIALS & METHODS

Samples

Flour samples were chosen from those studied previously (Chen and Hoseney, 1994). A flour sample (Table 1) that produced sticky doughs was designated as a sticky-dough flour control (SD). A commercial bread flour was designated as a nonsticky-dough flour control (NSD).

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Table 1—Effect of flour fractions on dough stickiness

Water insoluble	Water solubles	Dough stickiness[a]
NSD (Unfractionated)		32 ± 1
SD (Unfractionated)		106 ± 3
NSD	NSD	37 ± 1
NSD	SD	92 ± 3
SD	SD	111 ± 5
SD	NSD	42 ± 1

[a] gram-force, ± standard deviation

Dough stickiness, fractionation, and reconstitution

Dough stickiness was measured with the method described previously (Chen and Hoseney, 1994). The NSD and SD were dispersed in distilled water, at ambient temperature (flour:water = 1.3). Each mixture was stirred on a stir plate at moderate speed for 20 min and centrifuged (CU - 5000 Damon/IEC centrifuge) at 2000 × *g* for 30 min. The supernatant was separated from the centrifugate and collected as water solubles. The centrifugate was collected and labeled water insolubles. Both water-soluble and -insoluble materials were frozen and lyophilized. Doughs were mixed with the mixograph with four different combinations (Table 1): (1) water solubles from NSD with water insolubles from NSD; (2) water solubles from SD with water insolubles from SD; (3) water solubles from NSD with water insolubles from SD; and (4) water solubles from SD with water insolubles from NSD. Optimum mixing times and water absorption of doughs made with each combination were determined using a mixograph (Finney and Shogren, 1972).

Effect of dialyzed fractions on dough stickiness

Dialysis was used to fractionate the water-soluble fraction from the SD. A dialysis bag (Spectrapor membrane tubing) was used. The molecular weight cutoff was 6000 to 8000. The water solubles from SD were placed in the dialysis bag and dialyzed vs distilled water at 5°C for 24 hr. Both retentate and permeate were lyophilized. Doughs were made by optimally mixing the NSD with retentate or permeate.

Cation exchange

Cation exchange resin (CG-120 chromatographic grade resin, RSO-Na+ BIO-RAD Laboratories, Richmond, CA) was used to purify the permeate from SD collected from dialysis. The resin was first washed with 1N HCl and rinsed with four volumes of distilled water. Then permeate was introduced into the cation exchange column at 5 mL/min. The negatively charged and neutral fractions (N,−) that eluted from the cation exchange column were collected and lyophilized. Doughs were mixed optimally using NSD and the (N,−) fractions from the SD flour.

Anion exchange

Further separation of the negatively charged fraction from the neutral fraction was attempted using an anion exchange column. The anion exchange resin (AG 1-X8, chloride form, analytical grade BIO-RAD Laboratories, Richmond, CA) was washed with 1N NaOH and rinsed with four volumes of distilled water. Then the (N,−) fraction was introduced into the anion exchange column and the neutral fraction (N) was collected. The negatively charged fraction (−) bound to the resin was eluted from the column using 0.01N NaOH. The mixture was neutralized

Table 2—Effect of dialyzed fractions on dough stickiness

Flour	Added fraction	Dough stickiness[a]
NSD	None	32 ± 1
NSD	Retentate	36 ± 1
NSD	Permeate	87 ± 3

[a] gram-force, ± standard deviation

Table 3—Effect of ionic-exchanged fraction and NaOH on dough stickiness

Flour	Added fraction	Dough stickiness[a]
NSD	None	32 ± 1
NSD	Negative/Neutral	93 ± 3
NSD	Negative	32 ± 1
NSD	Neutral	36 ± 1
SD	None	106 ± 3
SD	0.01 N NaOH	45 ± 2

[a] gram-force, ± standard deviation

Table 4—Effect of peak materials from Bio-gels P10 and P30 on dough stickiness

Flour	Added fraction	Dough stickiness[a]
From Bio-gel P10		
NSD	None	32 ± 1
NSD	Negative/Neutral	92 ± 3
NSD	1st peak materials	91 ± 2
NSD	2nd peak material	36 ± 1
NSD	1st & 2nd peaks	94 ± 3
From Bio-gel P30		
NSD	Peak Material	89 ± 2

[a] gram-force, ± standard deviation

with HCl. The (N) and (−) fractions were lyophilized. Doughs were prepared with a mixograph using the NSD with the 2 fractions. Doughs were also prepared with a mixograph using SD flour with a 0.01N NaOH solution instead of water.

Gel filtration chromatography

Gel filtration was used to separate materials based on their size. Bio gel (P 10, BIO-RAD Laboratories, Richmond, CA) was packed in a column 60 cm in length and 2.5 cm diameter. A peristaltic pump P-1 (Pharmacia Fine Chemicals) was used for a flow rate of 36 mL/hr. A Fraction Collector Frac-100 (Pharmacia Fine Chemicals) was used with a fraction size of 6 mL. Total collected volume was 570 mL. This column was used to fractionate the neutral and negatively charged (N,−) fraction.

Carbohydrate analysis

The phenol/sulfuric acid method was used for carbohydrate analysis (Dubois et al., 1956). Absorbance for each fraction collected from the gel filtration experiment was measured at 490 nm on a spectrophotometer. Each fraction eluted from the gel filtration column also was tested for UV-absorbance at 280 nm.

Dough stickiness caused by gel filtration fractions

Two carbohydrate-containing peaks were obtained from the gel filtration column. The larger molecular weight peak also was UV-absorbing. Both peaks were collected separately and lyophilized. Doughs were prepared with a mixograph by mixing the NSD individually with the two samples.

P 30 Bio-gel filtration

Because the first peak on Bio-gel P10 was at or near the void volume, the larger molecular weight carbohydrate peak (containing the UV-absorbing material) was fractionated on a gel filtration column packed with Bio-gel P30. Column size and flow rate remained the same. The carbohydrate and UV-absorbance were monitored. The V_o and V_t were determined using blue dextran and glucose, respectively. Doughs were prepared with a mixograph by mixing the NSD with the peak materials.

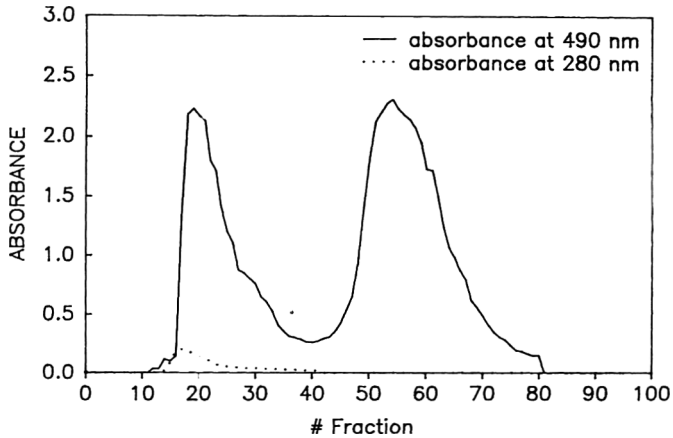

Fig. 1—Gel filtration chromatogram (Bio-gel P10) of the material containing the compound causing sticky dough.

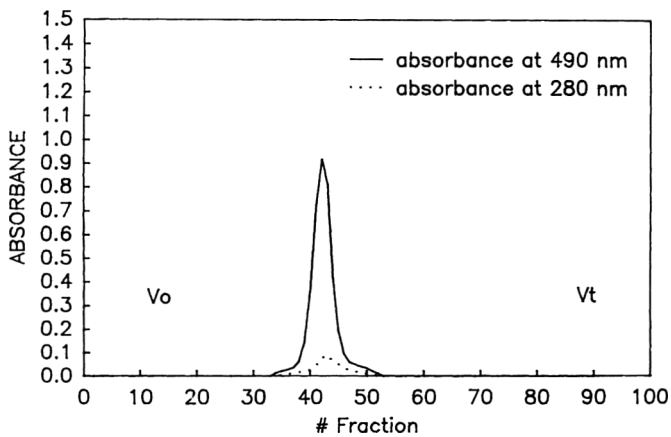

Fig. 2—Gel filtration chromatogram (Bio-gel P30) of the compound causing sticky dough.

NaOH treatment of the peak material

The peak materials were mixed with 0.01N NaOH. The mixture was allowed to stand for 15 min. The treated mixture was separated on the gel filtration column as reported previously (P30 Bio-gel). Carbohydrate and UV-absorbing materials were measured.

Purification of UV-absorbent peak material

The UV-absorbent peak material was analyzed in a high pressure liquid chromatograph (HPLC) model HP 1036 A (Hypersil ODS 5 μm column, 4.6 mm id * 100 mm with an Upchurch C-1 30B, 2 mm i.d. * 2 cm guard column). The procedure separates materials based on hydrophobicity. The mobile phase was a mixture of 35:65, methanol:citrate (pH 5.4). Flow rate was 1 mL/min. An Altex single piston pump and a 1036A UV detector (Hewlett Packard) were used.

Chemical composition of peak material

The UV-absorbent peak material was introduced into a HPLC Mass Spectrometer, mocel 5989A (Hewlett Packard). The HP 1050 series HPLC and HP 59980B particle beam LC/MS interface were used. The HPLC column was ODS Hypersil 5 μm, 100 * 2.1 mm, 799160 D-552. The mobile phase was 50:50, methanol:water. The flow rate was 0.5 mL/min. The mass spectrum of the UV-absorbent peak material was determined and compared to standards of glucose and ferulic acid.

Acid hydrolysis of carbohydrate peak material

The carbohydrate peak material was hydrolyzed with sulfuric acid for chemical composition analysis. The material was mixed with 2% sulfuric acid and held in a boiling water bath for 5 hr. The mixture then was cooled to room temperature (≈13°C) and introduced to the HPLC mass

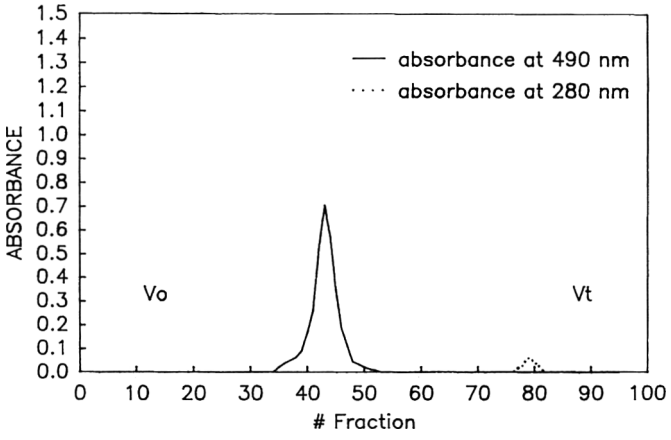

Fig. 3—Gel filtration chromatogram (Bio-gel P30) of the compound causing sticky dough after sodium hydroxide treatment.

Table 5—Effect of peak materials from Bio-gel P30 after NaOH treatment on dough stickiness

Flour	Added fraction	Dough stickiness[a]
NSD	None	32 ± 1
NSD	Negative/Neutral	92 ± 3
NSD	Carbohydrate peak	37 ± 1
NSD	UV absorbing peak	34 ± 1
NSD	Carbohydrate & UV	35 ± 1

[a] gram-force, ± standard deviation

spectrometer for chemical structure determination. Mass spectra of the mixture and of the glucose standard were compared.

RESULTS & DISCUSSION

Fractionation and reconstitution

Sticky doughs were produced by mixing the water solubles of SD with either its own water insolubles or with water insolubles of NSD (Table 1). Nonsticky doughs were produced when doughs were prepared from water solubles of NSD and its water insolubles or with the water insolubles of SD. This indicated that, to produce sticky dough, the water solubles of SD had to be present. When dough was made using NSD with the retentate fraction from dialysis, it was strong and not sticky. However, when the permeate fraction was mixed with NSD, a sticky dough was produced (Table 2). Thus, the substance causing sticky dough was in the permeate; the active substance was not a large polymer but was of relatively low molecular weight.

Cation and anion exchange

The permeate was subjected to cation exchange to separate positively charged (+) material from neutral and negatively charged fractions (N,−). Mixing the NSD with the (N,−) fraction resulted in sticky dough (Table 3). This indicated that the substance causing sticky dough was either neutral or negatively charged. To separate the negatively charged fraction from the neutral fraction, anion exchange was applied. Dough stickiness data (Table 3) showed that neither fraction caused dough stickiness. Because NaOH was the only chemical in the anion exchange process, we hypothesized that NaOH might have reduced dough stickiness. To test the effect of NaOH, doughs were prepared with a mixograph with SD and 0.01N NaOH. (Table 3). The dough stickiness was reduced sharply by the NaOH.

Gel filtration chromatography

Size exclusion or gel filtration was used to separate materials based on molecular sizes. Two peaks containing carbohydrate were detected (Fig. 1). A small peak, absorbing at 280 nm, coeluated with the first carbohydrate peak. Doughs were prepared with a mixograph by mixing the NSD separately with material from the two peaks. Material from the larger molecular weight peak produced sticky dough (Table 4).

P30 Bio-gel filtration experiment

Because the first peak on Bio-gel P 10 was at, or near, the void volume, the first carbohydrate peak material and UV-absorbent peak material were introduced into the gel filtration column packed with P30 Bio-gel. One peak containing carbohydrate and UV-absorbent materials was detected (Fig. 2). Doughs made by mixing the NSD with that material were sticky (Table 4).

A UV-absorbent peak (Fig. 3) was separated from the carbohydrate peak after NaOH treatment, indicating that NaOH had cleaved bonds between the two components. Doughs were prepared with a mixograph by mixing the NSD with either carbohydrate or UV-absorbent peak materials. Neither fraction produced sticky dough (Table 5). This indicated that the carbohydrate material and the UV-absorbing material had to be linked together to cause sticky dough.

Purification of UV-absorbent and carbohydrate peak materials

The HPLC chromatogram of the UV-absorbent peak material showed a single peak. Along with results from gel filtration (Bio-gel P30), this suggested that the UV-absorbing material was a single entity. The fraction was submitted to HPLC-mass spectrum analysis. The peak material was identified as ferulic acid by comparison with published library spectra and spectra of known ferulic acid (Fig. 4). The carbohydrate containing peak was hydrolyzed and submitted to mass spectral analysis. Comparison of the spectra with that of standard glucose indicated a hexose was the repeating unit of the carbohydrate material (Fig. 5).

Proposed model for the compound causing sticky dough

Based on our results, we propose that the substance causing sticky dough is a ferulic acid moiety attached to a carbohydrate chain through an ester bond. The carbohydrate chain length was determined by mass spectrometry to be ≈24. When such a compound was subjected to a basic pH environment, the ester bond was cleaved. The ferulic acid portion was separated from the carbohydrate portion. Once this separation occurred, the dough stickiness effect was reduced. In wheat flour ferulic acid is es-

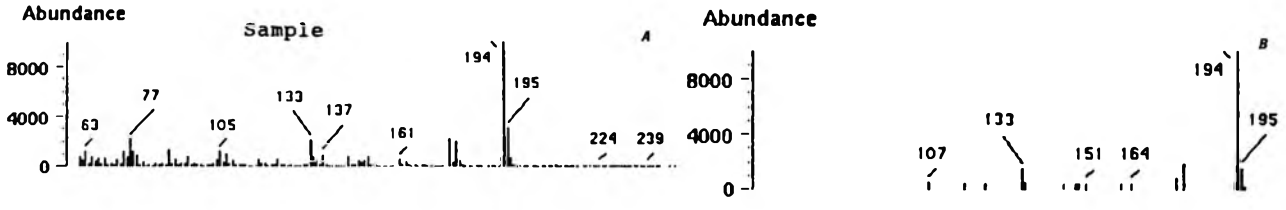

Fig. 4—(A) Mass spectra of UV-absorbent peak after saponification. (B) Mass spectra of ferulic acid.

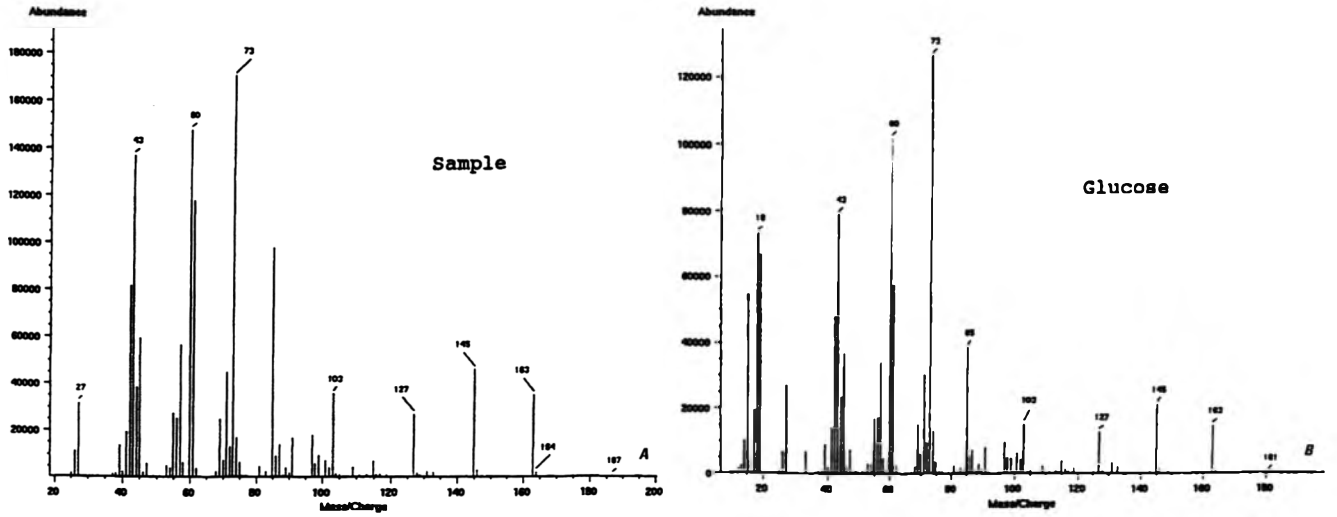

Fig. 5—(A) Mass spectra of hydrolyzed carbohydrate peak. (B) Mass spectra of authentic glucose.

terified to pentosans (Lineback and Rasper, 1988). However, the size of the carbohydrate entity was much larger than we found. No published reports were found showing esterification of ferulic acid to hexose chains.

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Photoacoustic Monitoring of Processing Conditions in Cooked Tortillas: Measurement of Thermal Diffusivity

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ABSTRACT

The thermal diffusivity and conductivity of cooked tortillas processed under different conditions were measured at room temperature using a proposed open-photoacoustic cell (OPC) detection technique. Samples were cut from cooked tortillas and exposed to air for different times prior to measurements. Variations in thermal diffusivity inversely correlated with cooking time. Lime appeared to increase the degree of crosslinking, raising thermal diffusivity and decreasing cooking time. By careful process modification a measure of control could be used to improve the heat conduction properties of tortillas.

Key Words: tortillas, photoacoustic, thermal diffusivity, conductivity.

INTRODUCTION

PHOTOACOUSTIC (PA) SPECTROSCOPY and related photothermal techniques (Rosencwaig, 1980; Vargas and Miranda, 1988) are well established spectroscopic techniques. PA technique, apart from providing direct optical absorption spectra (Lima et al., 1985), can also be used to perform depth profile analysis (Anjo and Moore, 1984; Nery et al., 1987) characterization of thermal properties (Adams and Kirkbright, 1977; Pessoa et al, 1986; Leite et al., 1987; Pinto Neto et al., 1990; Silva et al., 1993) and for investigation of the nonradiation relaxation process (Quimby and Yen, 1980; Lima et al., 1987). Also a quite substantial development of versatile and competitive instrumentation and experimental methodogies are suitable for daily practice.

PA directly monitors the heat generated in a sample, due to the nonradiative de-excitation processes, following the absorption of modulated light. In conventional experimental arrangements, a sample is enclosed in an air-tight cell and exposed to a chopped light beam. As a result of periodic heating of the sample, the pressure in the cell oscillates at the chopping frequency and can be detected by a sensitive microphone coupled to the cell. The resulting signal depends not only on the amount of heat generated (and, hence on the optical absorption coefficient and the light-into-heat conversion efficiency of the sample) but also on how the heat diffuses through the sample. The dependence upon these factors is the main reason for the versatility of the PA technique.

The quantity that measures the heat diffusion in the sample is called the thermal diffusivity α. Apart from its intrinsic importance, its determination gives the value of thermal conductivity k, if the density p and the thermal capacity c at constant pressure are known, since

$$\alpha = k/pc \qquad (1)$$

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The importance of α is that, like the optical absorption coefficient, it is unique for each material. Tabulated values of α were given (Touloukian et al., 1973), for a wide range of materials, such as, metals, minerals, foodstuffs, biological specimens. Thermal diffusivity is also known to be extremely dependent upon the effects of compositional and microstructural variables (Ziegler and Hasselman, 1981) as well as processing conditions for polymers (Torres Filho et al., 1989; Leite et al., 1987), ceramics (Ziegler and Hasselman, 1981) and glasses (Bento et al., 1987).

Thermal diffusivity can be accurately measured by PA and various arrangements have been developed. (Adams and Kirkbright, 1977; Pessoa et al., 1986). An improved gas-microphone detection technique, termed the open photoacoustic cell (OPC), specially suited for measuring thermal diffusivity has been proposed (Perondi and Miranda 1987). The method may be used either as a spectroscopic technique to observe chemical and structural changes (Cella et al., 1989; Marquenzini et al., 1990; Pereira et al., 1992) or to study thermal properties (Pinto Neto et al., 1990)

Our objective was to determine the potential of the OPC method for measuring thermal diffusivity of cooked tortillas. The thermal diffusivity of tortillas is important when predicting heat transfer rates during processing, and for process control.

MATERIALS & METHODS

Sample preparation

To demonstrate the potential of the technique, we made thermal diffusivity measurements on tortillas purchased from a commercial facility. Samples of tortillas were prepared from commercial instant corn flour, blue aleurone corn masa, wheat flour, instant whole corn flour made by extrusion (with and without lime), by cooking pieces of masa of circular shape on a hot plate. In addition, a commercial cooked tortilla (Tortiricas) was also studied. The tortilla was considered cooked when puffing occurred. Measured cooking times, except for the commercial cooked tortilla were compared (Table 1). Tortillas without lime do not puff in the usual way, so we defined the cooking time as the time required to observe the formation of small blisters. For determining cooking of tortillas with lime the tortilla was placed on a hot plate with repeated turning until it puffed. That moment was the cooking time. After being cooked, the tortillas were then exposed to air for periods of 4–7 days, to provide a hard consistency. Next, a small piece of tortilla was rubbed with sand-paper to provide samples with smooth and flat surfaces, with thickness from 100–300 μm. The structural and molecular changes of tortillas during drying, are (Tonella et al., 1983) dependent on both processing and storage conditions (Gómez et al., 1992). In addition the environment to which the samples were exposed also is important in determining the general condition of samples. Due to the extent of this "unknown prehistory" of our samples, neither water loss from samples nor variations in thickness and density were investigated in detail. The requirement of optical opacity (implicitly in the theoretical analysis) was realized by affixing a thin circular Al foil (20 μm-thick, 5 mm diam) to that side of the sample that received the radiation (affixed by means of a thin layer of vacuum grease).

Thermal diffusivity measurement: the open-cell detector

The cross section of the open-cell photoacoustic detector (Fig. 1) shows the mounting of the radiation absorbing material directly onto a commercial electret microphone. Clearly, such a PA detector requires

Table 1—Processing conditions as related to process and thermal parameters for tortillas

Processing conditions	Thickness (μm)	Cooking time (sec)	Exposure period (days)	Thermal capacity (J/m^3K)	Thermal diffusivity (m^2/sec)	Thermal conductivity (W/m K)
Commercial cooked tortillas	300	—	7	$(0.90 \pm .01) \times 10^6$	$(2.8 \pm .2) \times 10^{-7}$	$0.25 \pm .020$
Commercial corn flour	260	79	7	$(0.90 \pm .01) \times 10^6$	$(2.5 \pm .2) \times 10^{-7}$	$0.22 \pm .021$
Blue aleurone corn masa	200	62	4	$(1.00 \pm .01) \times 10^6$	$(4.7 \pm .2) \times 10^{-7}$	$0.47 \pm .025$
Wheat flour	170	60	4	$(0.95 \pm .01) \times 10^6$	$(4.1 \pm .2) \times 10^{-7}$	0.39 ± 0.195
Made by extrusion without lime	235	83	5	$(1.33 \pm .01) \times 10^6$	$(5.5 \pm .2) \times 10^{-7}$	$0.44 \pm .032$
Made by extrusion with lime	280	73	5	$(0.75 \pm .01) \times 10^6$	$(10 \pm .2) \times 10^{-7}$	0.75 ± 0.025

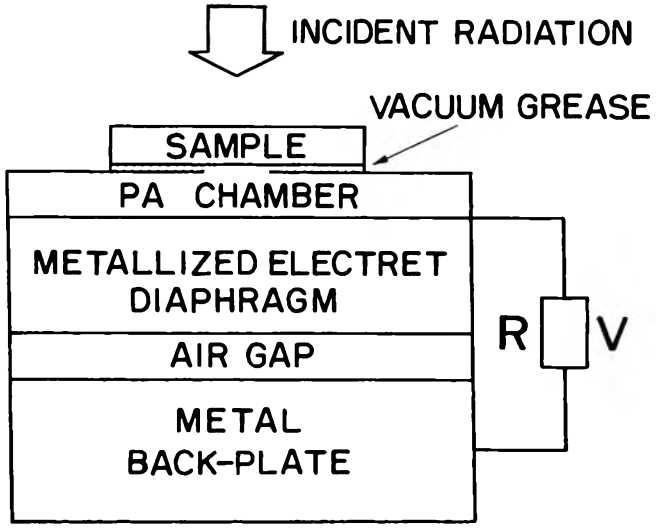

Fig. 1—Cross section of an open-cell photoacoustic detector.

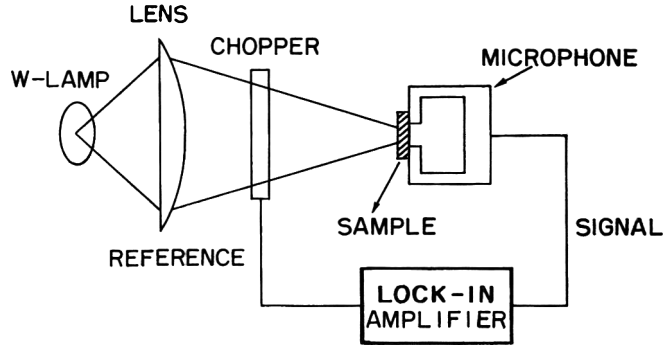

Fig. 2—Experimental arrangement used in OPC measurements.

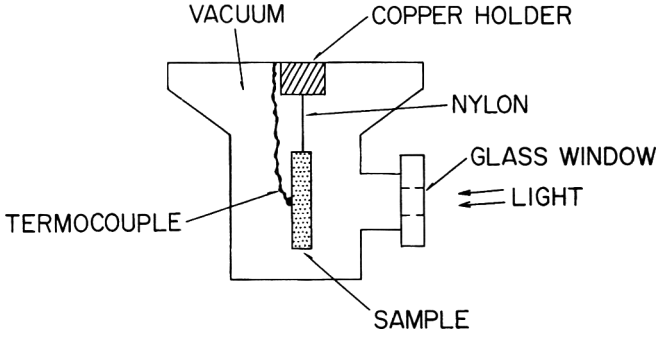

Fig. 3—Experimental arrangement used for thermal conductivity measurements.

neither additional transducing medium (i.e., the confined layer of air encountered with a conventional PA cell), nor time consuming machining of the cell since the microphone capsule acts simultaneously as a cell and as a detector. The typical design (Sessler, 1963; Sessler and West, 1973) of an electret microphone consists of a metallized electret diaphragm (12 μm Teflon with a 500–1000 ° thick deposited metal electrode) and a metal back-plate separated from the diaphragm by an air gap (45 μm). The metal layer and the back-plate are connected through a resistor R. The front sound inlet is a circular hole, 2mm diam, and the front air chamber adjacent to the metalized face of the diaphragm is ≈1 mm long. As a result of periodic heating of the sample by absorption of modulated light, the pressure in the front chamber oscillates at the chopping frequency causing diaphragm deflection, which generates a voltage V across the resistor R. This voltage V is subsequently fed into a FET pre-preamplifier built into the microphone capsule. The FET polarization voltage is usually between 1.5–3.0 V. The proposed OPC detector consisted of using the front chamber of the microphone itself as the gas chamber usual in conventional photoacoustics. The experimental arrangement (Fig. 2) consisted of an Oriel 250 W halogen lamp whose polychromate beam was mechanically chopped and focussed onto the sample. The sample was mounted in such a way as to cover the opening of the microphone. The signal from the microphone was connected to a lock-in amplifier (PAR, model 5210) used to register both signal amplitude and phase. These were recorded as a function of modulation frequency. This arrangement corresponded to a heat transmission configuration. That is, the deposited heat at the rear-side face of the sample (due to the light-into-heat conversion at the Al foil) diffused through the sample before reaching the PA chamber where it caused the pressure fluctuations that were detected by the microphone. The thermal wave attenuation in the sample was basically determined by the sample thermal diffusivity α.

Thermal conductivity measurements

The thermal conductivity, k, was measured using the temperature rise method (Hata, 1979; Mansanares et al., 1990), under continuous white light illumination. For the experimental arrangement (Fig. 3) samples were cut in the shape of squares (1 cm × 1 cm area) with both surfaces sprayed with black paint. A light-absorbing surface, and the same heat-transfer coefficient were thus assured for each side. The samples were adiabatically suspended in a Dewar flash which was subsequently vacuum sealed. Under these conditions the main heat-loss mechanism was by radiation. The Dewar had an entrance glass window through which the continuous white light beam was focused onto one of the sample surfaces. A thermocouple was attached on the opposite surface using thermal paste. In this way, the temperature evolution of the back surface could be monitored as a function of time. Care was taken to prevent the heating light from reaching the thermocouple. Since the sample thicknesses (typically on the order of 200 μm) were much smaller than their widths (e.g., 1 cm), the simple one-dimensional heat diffusion equation with radiation losses could be applied. Solving the one-dimensional heat diffusion equation (Carslaw and Jaeger, 1973) the long-term time evolution (i.e., for times greater than the heat diffusion time $\approx l^2/\alpha$, where ℓ is the sample thickness and α the thermal diffusivity) of the back surface temperature rise is given by:

$$\Delta T = \frac{I_o \alpha \tau}{\ell k}(1 - e^{-t/\tau}), \qquad (2)$$

where I_o is the intensity of the incident light beam and $\tau = lk/2\alpha H$ is the rising time. Here, $H = 4\sigma T_o^3$, where σ is the Stefan-Boltzman constant, T_o is the ambient temperature, and H is the radiation heat transfer coefficient.

RESULTS & DISCUSSION

THE BACK-SURFACE temperature increased on a 200-μm-thick, blue aleurone tortilla corn sample with time (Fig. 4a). The temperature cooling of the back surface also occurred when the illumination was switched off. The solid line represents the best fit of experimental data to Eq.(2) using τ as an adjustable pa-

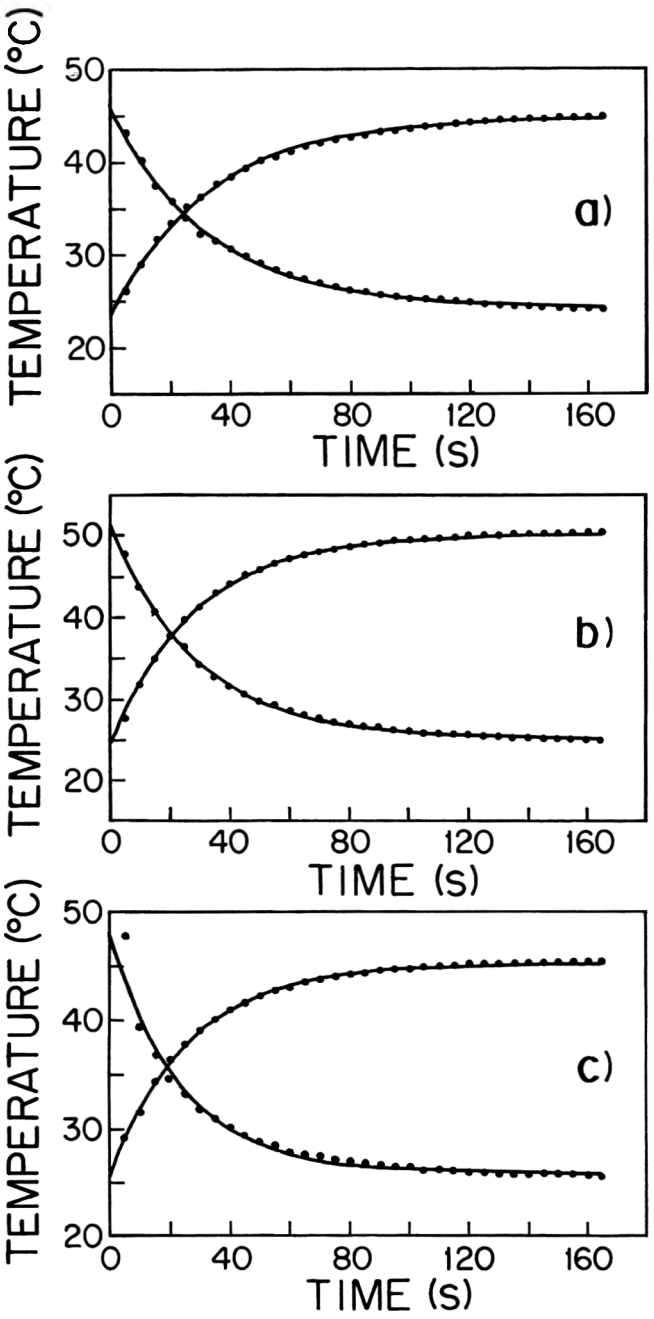

Fig. 4—(a) Back-surfaces temperature evolutions of a 200-μm-thick blue aleurone tortilla corn, (b) 235-μm-thick sample made by extrusion without lime, (c) 296-μm-thick sample made by extrusion, with lime, as a function of time under continuous white-light illumination. The solid lines represent the best fit of the experimental data to Eq.(2) of the text.

rameter. From the value of τ we got the experimental value of the thermal capacity, pc. We found $pc = 1.0 \times 10^6 J/m^3K$. The back surfaces of samples also had a temperature rise as a function of time for a 235-μm-thick tortilla samples made by extrusion without lime (Fig. 4b) and for the 296-μm-thick sample processed with lime (Fig. 4c). The temperature cooling of the back-surfaces, when illuminations was switched off is also shown. Repeating the same procedure as above, we got for the thermal capacity pc, the values, 1.33×10^6 J/m^3K for the sample without lime, and 0.75×10^6 J/m^3K for the sample with lime. The room-temperature values of the thermal capacity, processing conditions, cooking time and period of drying in air of the samples were compared (Table 1).

To understand the PA signal monitoring of the tortillas samples, using the open-cell detector, we must examine the modulation frequency dependence of the detected signal. As is well known (Vargas and Miranda, 1988), the usual way for extracting information on a sample's thermal diffusivity is to analyse data from plots of the magnitude and phase of the PA signal vs the modulation frequency. For the rear-side illumination configuration (Fig. 5) the thermal diffusion model (Rosencwaig and Gersho, 1976), predicts that the pressure fluctuation δp in the air chamber of the OPC detector is given by,

$$\delta p = \frac{\gamma P_o I_o (\alpha_g \alpha_s)^{1/2}}{2\pi l_g T_o k_s f} \frac{e^{j(\omega t - \pi/2)}}{\sinh(l_s \sigma_s)}, \qquad (3)$$

where f is the modulation frequency, γ is the air specific heat ratio, $P_o(T_o)$ is the ambient pressure (temperature), $l_g(\alpha_g)$ and $l_s(\alpha_s)$ are the length (thermal diffusivity) of the air and sample respectively, k_s is the thermal conductivity of the sample, $\sigma_s = (1 + j)a_s, a_s = (\pi f/\alpha_s)^{1/2}$ is the thermal diffusion length of the sample, $\omega = 2\pi f$ and $j = \sqrt{-1}$. In arriving at Eq.(3) we assumed that the sample was optically opaque and that the heat flux into the surrounding air was negligible. The implicit condition for optical opacity (meaning that all incident readiation was fully absorbed at the surface $z = -l_s/2$, was met by using a thin circular Al foil (20 μm-thick and 5 mm in diam) attached to the front of the sample by a thin layer of vacuum grease. The thermal diffusion time $\pi l^2/\alpha$ was on the order of 13.6μs ($\alpha_{Al} = 9.2 \times 10^{-5}$m^2/sec), so that the heat generated in the absorber Al foil was assumed to be instantaneously transmitted to the sample. Equation (3) was further simplified in two limiting cases depending upon thermal properties of the samples. For a thermally thin sample, namely $l_s a_s \simeq 1$, Eq(3) reduces to

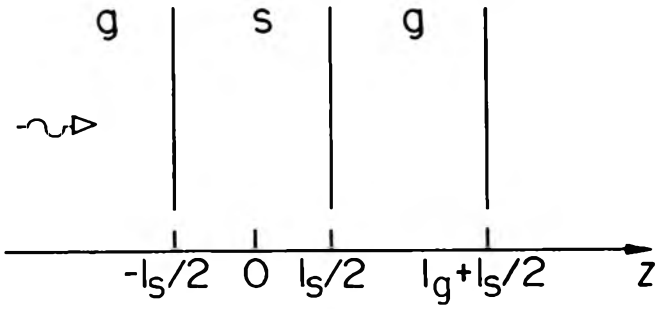

Fig. 5—Schematic geometry for the PA signal generation in which the incident light beam of intensity I_o is fully obsorbed at $z = -l_s/2$.

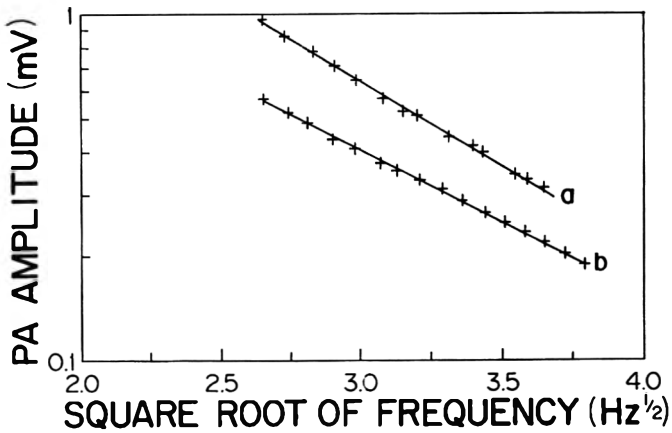

Fig. 6—Dependence of the PA signal amplitude as a function of the frequency square root for (a) the 300μm-thick sample (commercial cooked tortilla) and (b) the 260 μm-thick sample (prepared form commercial instant corn flour). The solid curves represent the fit of the experimental data to Eq. (6).

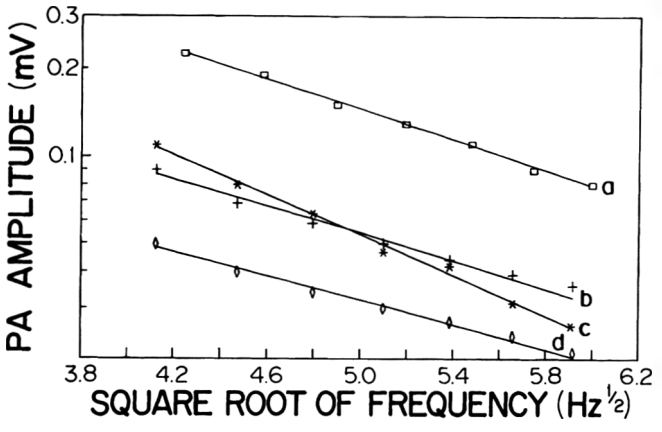

Fig. 7—Dependence of the PA signal amplitude as a function of the frequency square root of samples processing with (a) blue aleurone corn masa, (b) wheat flour, (c) made by extrusion without lime (d) made by extrusion with lime. The solid curves represent the fitting of the experimental data to Eq.(6).

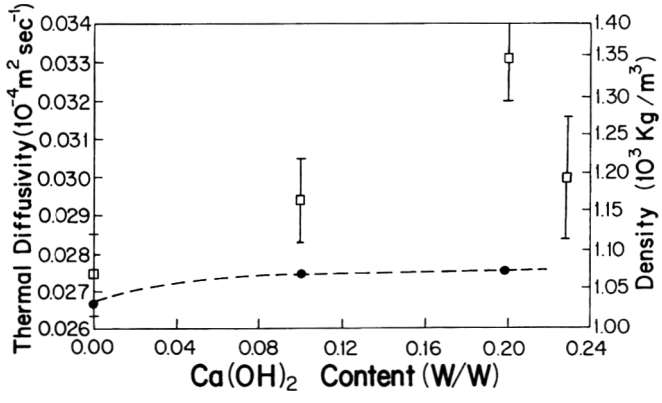

Fig. 8—Thermal diffusivity of tortillas made from corn by nixtamalization process as a function of Ca(OH)$_2$ concentrations. The dotted line represents the density.

$$\delta p = \frac{\gamma P_o I_o \alpha_g^{1/2} \alpha_s}{(2\pi)^{3/2} T_o l_g l_s k_s} \frac{e^{j(wt-3\pi/4)}}{f^{3/2}} \tag{4}$$

That is, the PA signal amplitude decreases as $f^{-1.5}$ as we increased the modulation frequency. In contrast, if the sample was thermally thick, namely, if $l_s a_s \simeq 1$, we get from (3),

$$\delta p \cong \frac{\gamma P_o I_o (\alpha_g \alpha_s)^{1/2}}{\pi T_o l_g k_s} \frac{\exp[-l_s(\pi f/\alpha_s)^{1/2}]}{f} e^{j(wt-\pi/2-l_s\alpha_s)} \tag{5}$$

Equation(5) indicates that, for a thermally thick sample, the amplitude of the PA signal decreases exponentially with modulation frequency according to $(1/f)\exp(-a\sqrt{f})$, where $a = (\pi l_s^2/\alpha_s)^{1/2}$. The thermal diffusivity α_s, can then be obtained from the signal amplitude data, as a function of modulation frequency, by fitting it to the expression,

$$S = (A/f)\exp(-\alpha\sqrt{f}). \tag{6}$$

The constant A, in the measured signal S, apart from geometric factors, includes all other factors such as gas thermal properties. We thus have two adjusting parameters A and a to describe the PA monitoring of thermal diffusivity of a sample. Knowing the coefficient a from the fitting procedure, α_s is readily obtained from $\alpha_s = \pi(l_s/a)^2$.

The PA signal amplitude as a function of the frequency square root was compared (Fig. 6) for the (a) 300 μm-thick sample (commercial cooked tortilla) and (b) 260μm-thick sample prepared from commercial instant corn flour. Both samples were exposed to air over 1 wk. The solid curves represent the fitting

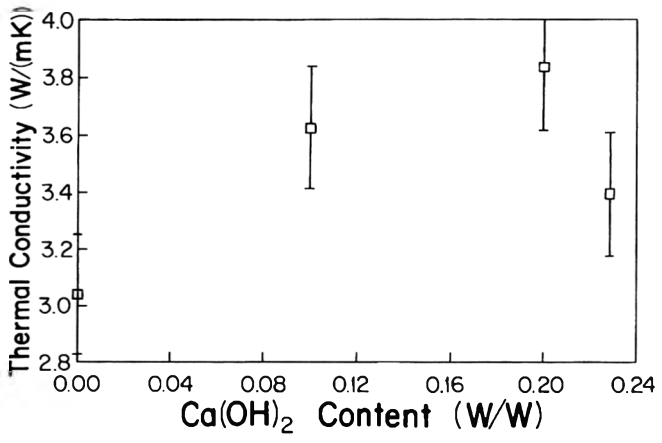

Fig. 9—Thermal conductivity as a function of Ca(OH)$_2$ concentration of the tortillas made by nixtamalization process.

of experimental data to Eq. (6). The resulting values of α from the data fitting was $\alpha = (2.8 \pm .2) \times 10^{-7}$m^2/sec for the commercial cooked sample and $(2.5 \pm .2) \times 10^{-7}$m^2/sec for the commercial corn flour sample. Note that, in the frequency range (5–50 Hz) of our experimental results [typical value for thermal diffusivity of a food specimen ($\alpha = 10^{-7}$m^2/s)], samples were thermally thick i.e., their thermal diffusion lengths $(\alpha/\pi f)^{1/2}$ were much smaller than their thickness.

The same procedure was applied to other samples. The PA signal amplitude of samples as a function of frequency square roots were compared (Fig. 7) for tortillas processed with (a) blue aleurone corn masa (b) wheat flour, (c) made by extrusion without lime, and (d) made with lime. The solid curves represent fitting of the experimental data to Eq.(6). The thermal conductivity was determined using the measured values of thermal capacity pc and the previously determined values of α.

The magnitudes of thermal parameters (Table 1), were in the range of those obtained by the line source technique on grain dust of wheat and corn (Chang et al., 1980) and on Mexican tortilla dough, using a split-split unit experiment (Griffith, 1985). There was also a consistent trend, suggesting that the values of thermal diffusivity of the tortillas was inversely correlated with cooking time. This reflects unequivocally the physical meaning of thermal diffusivity (α). It is essentially a measure of thermalization time within a given sample; the greater the thermal diffusivity the smaller the thermalization time.

As processing conditions changed (Table 1) the thermal parameters also increased, until they reached a value around 10×10^{-7} m^2/seg and 0.75W/m K respectively for thermal diffusivity and thermal conductivity of a sample prepared with lime, Ca(OH)$_2$. These values were larger than the thermal parameters of tortillas prepared without lime. To explain this, we need to know whether the sample densities varied, but such measurements were not studied in the set of samples. However, as a final check we measured the thermal diffusivity and thermal conductivity of tortillas made from corn by the nixtamalization process (Serna-Saldívar et al., 1990). Here the processing conditions were controlled, and involved alkaline cooking, washing, density, thickness texture of masa, viscosity, size and shape of granules. The experimental values of thermal diffusivity (Fig. 8) and thermal conductivity (Fig. 9) as a funtion of lime concentration were compared. The experimental points represent the best fit of experimental data to Eq. (6) and Eq. (2), respectively. After a specific concentration of Ca(OH)$_2$, there was a sharp decrease in both thermal diffusivity and thermal conductivity. The maximum thermal parameters were reached at about 0.20 of Ca(OH)$_2$ and this concentration is commonly used in practice to achieve optimum cooking of tortillas. To explain the results, we checked whether the sample density varied. The density p remained constant (1.03×10^3Kg/m^3) in the untreated sample, i.e., without lime, and as the crosslinking reaction began, p

tended to increase a little, from ($1.03 \times 10^3 Kg/m^3$) to a value constant of ($1.07 \times 10^3 Kg/m^3$).

Thus the observed increase of thermal diffusivity with lime, may then be attributed to changes in values of thermal conductivity k and specific heat c as a function of concentration of $Ca(OH)_2$. The specific heat was not expected to change much as the crosslinking reaction occurred. The values of specific heat c, for liquids and solids were, in general, in the same range. However, the same was not true regarding thermal conductivity, for which the difference between values for solids and liquids may be several orders of magnitude. We thus, attributed the changes in α, (Fig. 8) to an increase in thermal conductivity of tortillas as crosslinking proceeded. Enhancement of the crosslinking degree in the samples was expected to increase thermal conductivity, as with an increase in crystallinity. The overall shape of the thermal diffusivity showed a very close resemblance to that of viscosity (Fanconi et al., 1986). This was consistent with the intuitive expectation that thermal diffusivity should change similarly to viscosity; the higher the thermal diffusivity the higher the viscosity should be. Also it was supporting the suggestion that an increment in the viscosity (or diffusivity) was due to calcium crosslinking, keeping starch granules and proteins from swelling and collapsing (Tonella et al., 1983).

CONCLUSIONS

A NOVEL OPEN-CELL photoacoustic (OPC) technique was useful for obtaining thermal diffusivity of tortillas samples, prepared under different conditions. A relatively simple experimental arrangement enabled discrimination of tortillas specimens by means of thermal diffusivities, and may serve as a quality indicator for the product. The values of α, and k will be of interest, for theoretical modeling of thermal processing for optimal cooking and heat treatments of tortillas.

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Characteristics of Acid Hydrolysate from Defatted Peanut Flour

RUDEEPAN WATTANAPAT, TOMMY NAKAYAMA, and LARRY R. BEUCHAT

ABSTRACT

We hypothesized that aflatoxin-contaminated defatted peanut flour could be acid hydrolyzed to produce an aflatoxin-free seasoning sauce. Physical and chemical properties and amino acid composition of the flour hydrolyzed at 120°C for 4 hr with 5N HCl and destruction of aflatoxin B_1 during hydrolysis were investigated. The pH (5.72), specific gravity (1.20), total solids (402 mg mL^{-1}), and reducing sugar (2.9 mg mL^{-1}) and total nitrogen (17.5 mg mL^{-1}) contents of the peanut hydrolysate were similar to those of commercial soy sauces or seasoning sauces made by chemical processes. Glutamic acid, aspartic acid, arginine and glycine were the major amino acids in the hydrolysate and should contribute substantially to enhancing flavor. Aflatoxin B_1 was totally destroyed during acid hydrolysis at 100 or 120°C.

Key Words: acid hydrolysate, defatted peanut flour, aflatoxin degradation

INTRODUCTION

HYDROLYZED VEGETABLE PROTEINS (HVP) produced by acid hydrolysis of plant proteins are used in food products to enhance taste and impart meat-like and other flavors. Such hydrolysates have unique flavors and flavor enhancing characteristics due to their content of salt, amino acids, peptides, and other components. Their flavor characteristics can differ somewhat, depending on the nature of starting materials, conditions of hydrolysis and post hydrolysis treatment.

Hydrolyzates have been consumed for hundreds of years as traditional soy sauce where flavor development comes from fungal hydrolysis of soybean components and varying amounts of adjuncts, principally wheat. Refinements to the traditional process include use of selected cultures, pasteurization, and other process improvements to yield consistent products in less time.

The process of producing plant protein hydrolysates using strong acids has been applied for about 100 years (Manley et al., 1981). Acid hydrolysates can be produced from any edible protein source by treating proteins with mineral acid at elevated temperature, subsequent neutralization with alkali, and removal of insoluble materials. Commercial HVP can be made from any highly proteinaceous material such as defatted oilseeds or cereals of which defatted soybean, wheat, and corn gluten are the most common. Factors on which selection of raw materials is based, for the production of HVP are cost and chemical, physical, sensory, and toxicological properties (Olsman, 1979; Dzanic et al., 1985).

Peanut meal from peanut oil production, because of its relatively low cost, high protein and low fat is a potential raw material for HVP production. The effects of process variables on acid hydrolysis of defatted peanut flour were reported by Wattanapat et al. (1994), but physical and chemical characteristics of the product have not been reported.

Strong acids have been reported to catalyze conversion of aflatoxin B_1 and G_1 to B_{2a} and G_{2a}, which are less toxic than their original source compounds (Dutton and Heathcote, 1968; Pohland et al., 1968; Pons et al., 1972). Williams and Dutton (1988) demonstrated that the production of HVP from contaminated defatted peanut meal by acid hydrolysis, under conditions simulating a commercial process, completely destroyed and removed aflatoxins. Since peanut meal may be contaminated with aflatoxins, the toxicological quality of HVP made from peanut meal also needs to be examined.

Our objective was to determine physical and chemical properties of acid hydrolysates made from defatted peanut flour and to confirm the destruction of aflatoxin B_1, (normally the major aflatoxin produced by aflatoxigenic molds) as affected by various acid hydrolyses. The product was compared to commercial soy or seasoning sauces made by chemical hydrolysis.

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MATERIALS & METHODS

Materials

Defatted peanut flour was prepared as described (Wattanapat et al., 1994). Commercial soy sauces and seasoning sauces purchased from a local market were also analyzed.

Hydrolysis process

Defatted peanut flour (25g) was mixed with 100 mL of 5N HCl in a 237-mL bottle. Bottles were tightly sealed with a crown cap and placed in an oil bath at 120°C. After 4 hr hydrolysis, bottles were cooled to room temperature (≈21°C). Hydrolyzed product was immediately neutralized with Na_2CO_3 to pH 5.5-6.0 and filtered under vacuum through Whatman No. 1 paper to remove insoluble materials. The filtrate was treated with activated carbon (0.1%, w/v) at 50°C for 2 hr, and then filtered. Hydrolysate was aged for 1 wk at room temperature before being evaluated.

Methods of analysis

Total nitrogen was determined by the macro-Kjeldahl method (AOAC, 1984). **Sodium chloride** content of test products was determined according to the Volhard method (Egan et al., 1981). **Total solids** were determined by evaporating 10 mL of sample in an aluminum vessel and drying at 70°C under vacuum for 12 hr. Samples (2.5 mL) were diluted to 50 mL with deionized water and **reducing sugar content** was determined by the dinitrophenol method of Ross (1975) with modifications as described by Wattanapat et al. (1994). **Specific gravity** of hydrolysates was determined with a hydrometer, and **pH** was measured with a Acumet pH meter (model 630, Fisher Scientific, Pittsburgh, PA). **Amino acid profiles** of peanut butter hydrolysates and commercial products were determined by HPLC (Waters Associates, Milford, MA). Samples (2.5 mL) were diluted to 50 mL with deionized water. Deproteinization and amino acid analysis were done following the procedure described by Wattanapat et al. (1994).

Statistical analysis

Data were analyzed using the Statistical Analysis System (SAS Institute, Inc., 1985). Differences in mean values between individual treatments were determined by the Least Significant Different (LSD) test at $P < 0.05$.

Preparation of aflatoxin contaminated flour

Defatted peanut flour (400 g) was mixed with 100 mL of chloroform containing 400 µg aflatoxin B_1 (Sigma, St. Louis, MO) in an amber glass

Table 1—Physical and chemical properties of peanut protein hydrolysate and commercial seasoning sauces[a]

Product	pH	Specific gravity	Total solids (mg mL^{-1})	Reducing sugars (mg mL^{-1})	Total nitrogen (mg mL^{-1})	Sodium chloride (mg mL^{-1})
Peanut hydrolysate	5.72 ± 0.04a	1.20 ± 0.00b	402 ± 3.75bc	2.9 ± 0.00c	17.5 ± 0.07b	239 ± 1.27a
Commercial 1[b]	4.81 ± 0.02c	1.19 ± 0.01c	377 ± 11.10c	87.2 ± 1.13a	14.3 ± 0.78c	180 ± 2.97c
Commercial 2[b]	4.65 ± 0.04d	1.21 ± 0.00b	424 ± 0.42b	60.4 ± 1.70b	15.2 ± 0.35c	210 ± 0.21b
Commercial 3[c]	5.19 ± 0.08b	1.24 ± 0.01a	517 ± 16.40a	3.4 ± 0.17c	31.2 ± 1.27a	214 ± 9.19b
Commercial specification[d]	4.5–6.0	—	> 320	< 100[e]	> 7.2	130–210

[a] Mean values of two replicates (± standard deviation) followed by the same letter in a column are not significantly different ($P \geq 0.05$).
[b] Commercial soy sauce.
[c] Seasoning sauce made from hydrolyzed plant protein.
[d] From U.S. General Services Administration (1983).
[e] Invert sugar.

Table 2—Amino acid composition of peanut hydrolysate and commercial products (% of total by weight)[a]

Amino acid	Peanut hydrolysate	Commercial product 1[b]	Commercial product 2[b]	Commercial product 3[c]
Aspartic acid	13.55 ± 0.06a	13.21 ± 0.35ab	12.57 ± 0.14b	10.06 ± 0.55c
Threonine	2.86 ± 0.04d	3.59 ± 0.07b	3.25 ± 0.07c	3.80 ± 0.10a
Serine	6.06 ± 0.05b	4.25 ± 0.08c	3.52 ± 0.34d	6.61 ± 0.06a
Glutamic acid	22.37 ± 0.26b	18.95 ± 0.00c	18.67 ± 0.66c	27.37 ± 0.77a
Proline	5.67 ± 0.09b	9.07 ± 2.51ab	6.06 ± 0.76b	11.03 ± 0.38a
Glycine	7.24 ± 0.17a	4.22 ± 0.07c	5.11 ± 0.07b	3.98 ± 0.32c
Alanine	4.08 ± 0.10c	9.78 ± 0.28a	7.98 ± 0.34b	8.35 ± 0.53b
Cystine	ND[d]	ND	ND	ND
Valine	2.50 ± 0.42b	3.74 ± 0.18a	3.98 ± 0.14a	2.92 ± 0.72ab
Methionine	0.44 ± 0.08a	0.61 ± 0.28a	0.86 ± 0.04a	0.96 ± 0.35a
Isoleucine	2.47 ± 0.04b	2.93 ± 0.22b	4.16 ± 0.23a	1.73 ± 0.23c
Leucine	6.82 ± 0.10a	6.84 ± 0.30a	7.16 ± 0.06a	3.99 ± 0.11b
Tyrosine	2.29 ± 0.13ab	2.83 ± 0.12a	2.18 ± 0.03b	0.91 ± 0.41c
Phenylalanine	5.08 ± 0.16a	5.08 ± 0.36a	4.64 ± 0.15a	4.64 ± 0.46a
Lysine	3.80 ± 0.07c	5.41 ± 0.65b	7.47 ± 0.40a	4.32 ± 0.21bc
Ammonia	2.11 ± 0.04c	3.76 ± 0.28a	2.86 ± 0.07b	2.46 ± 0.16bc
Histidine	2.33 ± 0.20a	1.98 ± 0.46a	2.49 ± 0.36a	2.29 ± 0.32a
Arginine	10.34 ± 0.08a	3.76 ± 0.26d	7.05 ± 0.37b	4.57 ± 0.01c

[a] Mean values of two replicates (± standard deviation) followed by the same letter in a row are not significantly different ($P \geq 0.05$).
[b] Commercial soy sauce.
[c] Seasoning sauce made from hydrolyzed plant protein.
[d] None detected.

jar. The chloroform was removed by placing the opened jar in the air flow of a fume hood overnight. Flour was thoroughly mixed by tumbling in a jar overnight. The flour was stored at 5°C until used.

Hydrolysis of contaminated flour

Hydrolysis conditions. Flour containing aflatoxin B_1 was hydrolyzed at 100°C and 120°C for 0, 4, and 8 hr using 3N and 5N HCl. Additional samples treated with water, 3N, and 5N HCl for 0 hr at room temperature (21°C) were also prepared. Experiments were replicated twice. Duplicate samples were analyzed at each hydrolysis time.

Hydrolysis procedure. Flour (2.5 g) was mixed with HCl (10 ml) in a tightly sealed screw-top culture tube and placed in an oil bath (Thermomix 1480, B. Braun, Germany) where temperature was controlled ±0.1°C. When the hydrolysis temperature was attained (3 min at 100°C and 10 min at 120°C), tubes were removed after 0, 4 and 8 hr, and cooled to room temperature.

Aflatoxin extraction and quantification

Hydrolysate was transferred to a 150-mL beaker and diluted with 50 mL of 16% (w/v) NaCl solution before neutralizing with Na_2CO_3 to pH 5-6. To provide ≈ 70% methanol in the final extraction medium, neutralized hydrolysate was diluted with methanol to 200 mL in a volumetric flask. The mixture was stirred vigorously using a magnetic stirrer for 15 min, and filtered through a fluted filter paper (Whatman No. 1) before combining 15 mL of filtrate with 30 mL water. Samples were passed through an Aflatest-P affinity column (Vicam, Somerville, MA). Because the accuracy of the column was limited to quantities of < 50 ng of aflatoxin, the volume of sample applied varied from 5–15 mL according to the concentration of aflatoxin determined from preliminary analysis. The column was washed twice with 10 mL of water. The aflatoxins were then eluted with 1 mL of HPLC grade methanol and collected in 4-mL amber vials. Methanol was evaporated under a stream of nitrogen. Samples were dissolved in 2 mL of methanol-water (1:1, v:v) and analyzed (20 µL) for aflatoxin content using the HPLC method described by Clavero et al. (1993). An aflatoxin solution containing 10 ng mL^{-1} B_1, 10 ng mL^{-1} G_1, 3 ng mL^{-1} B_2 and 3 ng mL^{-1} G_2 prepared from standard stock solutions containing 5 µg B_1, 5 µg G_1, 1.5 µg B_2 and 1.5 µg G_2 per mL in benzene-acetonitrile (98:2, v/v) was used to calibrate a standard curve. Extraction and quantification of aflatoxin B_1 were done under dim light.

RESULTS & DISCUSSION

Physical and chemical properties

Physical and chemical properties of peanut protein hydrolysate and commercial products were compared (Table 1). Characteristics of commercial products varied but were in the range of commercial specifications for nonfermented soy sauce (U.S. General Services Administration, 1983). The NaCl content ranged from 180–214 mg mL^{-1} and pH from 4.7–5.2. Products with high total solids, which depends on salt and other compounds, were also high in specific gravity. Both characteristics influence the texture of such hydrolyzed products.

The peanut hydrolysate had chemical properties similar to those of the commercial products. The NaCl content and pH of peanut hydrolysate were slightly higher and the reducing sugar content was lower. The pH, which could be adjusted during neutralization, influences flavor and product stability. The NaCl in the hydrolyzed product was a result of neutralization with Na_2CO_3, which could be controlled to some extent by changing the amount of HCl used. Other ways to reduce NaCl include dilution with water or removal by dialysis. The NaCl content should be kept high enough to be effective for microbiological stability. The relation between sodium intake and hypertension has necessitated reduction in sodium consumption by some people and must be considered in terms of dietary constraints.

Commercial products 1 and 2 were high in reducing sugars, which contributed to their sweet taste. We observed that acid hydrolysis causes extensive destruction of reducing sugars (Wat-

tanapat et al., 1994). The high concentration of reducing sugars in the commercial products suggests that sugar or corn syrup may have been added. Total nitrogen content can be an indicator of product quality and concentration of amino compounds. The total nitrogen content of the peanut hydrolysate was in the range of the commercial soy sauces.

Amino acids are major components that contribute to taste and flavor enhancing properties of protein hydrolysates. Amino acid or amino nitrogen is considered a major criterion for judging quality of hydrolysates. The higher the amino acid or amino nitrogen content, the higher the quality (Hall, 1946). The characteristic flavor of hydrolysates varies according to amino acid composition. Hydrolysates which contain a large number of amino acids have preferred quality ratings, which indicate more satisfying flavors and more acceptable tastes than those containing one or only a few amino acids.

The ratio of amino acids in hydrolysates varied with the protein material source (Table 2). Amino acid composition of peanut hydrolysate was different from that of soy sauces. Nonessential amino acids such as aspartic acid, glutamic acid, arginine, and glycine were the major amino acids in peanut hydrolysate. The high amount of dicarboxylic amino acids and glycine may enhance the flavor of hydrolysates. Dicarboxylic amino acids, especially glutamic and its mono sodium salt, are important to the flavor enhancing properties of HVP products (Manley et al., 1981). Glycine, which has a sweet taste (Kirimura et al., 1969; Solms, 1969), might contribute to sweetness of the products. Arginine, which is reported to be bitter and slightly sweet (Kirimura et al., 1969), on the other hand, may lower the sensory quality of hydrolysates.

Other hydrophobic amino acids such as leucine and phenylalanine may impart a slightly bitter taste (Kirimura et al., 1969; Solms, 1969). However, the taste of a product cannot be judged on the basis of the inherent taste of individual amino acids. Taste quality of amino acids may vary with their concentration (Solms, 1969). The contribution of amino acids to flavors of a food product can differ from the flavors of the pure compounds because of interactions, i.e., synergism or potentiation (Amerine et al., 1965; Solms, 1969).

Destruction of aflatoxin B_1 as affected by acid hydrolysis process

The recovery of aflatoxin B_1 from peanut flour at room temperature without acid treatment averaged 85%. The reduced recovery rate was attributed to inefficiency of extraction. Data clearly showed that strong acid effectively degraded aflatoxin B_1. It was decreased by 78% and 99.6% when contaminated flour was treated with 3N and 5N HCl, respectively, at room temperature. Complete destruction of aflatoxin B_1 occurred in all samples treated with acid at 100 and 120°C. These results confirmed the report of Williams and Dutton (1988) and indicate that acid hydrolysates from defatted peanut flour would be devoid of safety problems associated with aflatoxins. These results also suggest that aflatoxin-contaminated peanut meal or flour (usually unacceptable for use in food or feed) may be used for safe production of HVP.

CONCLUSION

HVP from acid hydrolysis of defatted peanut flour had chemical and physical properties similar to commercial nonfermented soy sauces and seasoning sauces. The amino acid composition of peanut hydrolysate was different from commercial products. Peanut hydrolysate contained aspartic acid, glutamic acid and arginine as major amino acids. The flavor quality of the product would depend on amino acid composition and other factors and must be examined through sensory evaluation.

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Bacillus cereus and *Bacillus stearothermophilus* Spore Inactivation in Batch and Continuous Flow Systems

G.G. WESCOTT, T.M. FAIRCHILD, and P.M. FOEGEDING

ABSTRACT

Thermal inactivation kinetics were evaluated in batch and continuous flow systems using phosphate buffer between 99 and 107°C for *Bacillus cereus* T and 128.5 and 139°C for *Bacillus stearothermophilus* ATCC 12980 spores. z_D-Values for *B. cereus* spores in batch and continuous flow systems were not significantly different; the continuous flow system was more lethal than the batch system. z_D-Values obtained using *B. stearothermophilus* spores in batch and continuous flow systems were different; the batch system was more lethal than the continuous flow system. Thus, use of batch generated data to predict or design continuous flow processes may not be accurate.

Key Words: *Bacillus cereus*, *Bacillus stearothermophilus*, spore inactivation, continuous flow

INTRODUCTION

FOODS OFTEN ARE PASTEURIZED or sterilized by continuous thermal processes. Microbiological characterization of systems and processes is important to validate lethality. Continuous ultra-high temperature (UHT) processes are difficult to characterize because of problems in achieving the high temperatures and short times in a laboratory for laboratory modeling of such systems. The large size and hazards of contamination often preclude use of commercial UHT systems for determination of microbiological inactivation kinetics (Davies et al., 1977). The large number of spores required for commercial systems are laborious to prepare and may introduce problems of spore aging and batch consistency (Franklin, 1970). Therefore, batch measurements of microbial inactivation at relatively low temperatures typically have been used to estimate lethality of industrial continuous flow processes at higher temperatures. Extrapolation of batch data is often outside the temperature range at which it was generated. Using batch data to design continuous flow systems may not be accurate and may result in under- or over-processing to assure safety and/or sterility. Laboratory scale thermal processing systems have been designed and built at North Carolina State University to allow kinetic data generation in a continuous flow system. One such system was used to generate thermal destruction/inactivation data for *Listeria innocua* (Fairchild et al., 1994) and acid hydrolysis of sucrose (Miles, 1993).

Conflicting results for thermal inactivation in batch and continuous flow systems can be compounded by the method of processing, suspending medium, and the organisms evaluated. Burton et al. (1977) reported that less than satisfactory sterilizing effects were frequently obtained in practical UHT sterilizers than were expected from capillary tube experiments. They compared batch and continuous flow thermal processing using *B. stearothermophilus* spores heated in water and milk. They used a capillary tube batch heating system, a laboratory scale direct heating UHT sterilizer (infusion-type for processing small batches) and a larger scale direct heating ultra high temperature sterilizer (continuous steam injection). Using milk, the capillary tube method gave lower D-values than did either the batch infusion sterilizer or continuous steam injection sterilizer at 138 to 145°C. Agreement between the systems was better in water than in milk.

Fairchild et al. (1994) compared batch and continuous flow thermal inactivation systems using *Listeria innocua* in raw milk. A fluid flow capillary tube reactor was used to expose sealed capillary tubes to high velocity water flow. A laboratory scale thermal processing system was used to generate continuous flow thermal inactivation data. The z_D-values were different between batch and continuous flow systems suggesting that the mechanism of thermal destruction may be different. The decimal reduction time curves were coincident at 69°C, while below 69°C, the continuous system predicted a more lethal process. Above 69°C, the batch system predicted a more lethal process.

In continuous flow systems, the temperature through the hold tube decreases due to heat loss. The equivalent point method (EPM) has been useful for kinetic data generation in continuous flow systems (Swartzel, 1984). The EPM is a procedure to determine reaction kinetics in fluids during nonisothermal continuous flow. This procedure allows kinetic data generation for thermal destruction at processing conditions simulating actual conditions (velocities, Reynolds number, shear stress at wall, etc.). The EPM has been successfully applied using chemical systems (Sadeghi and Swartzel, 1990; Miles, 1993) but has not been evaluated with biological organisms.

Our objective was to compare kinetics generated in continuous flow inactivation with those from a traditional batch method. Spores of mesophilic (*Bacillus cereus* T) and thermophilic (*Bacillus stearothermophilus* ATCC 12980) bacteria were used to address these objectives and allow comparisons over two temperature ranges. The EPM was applied to evaluate kinetic data from the continuous flow systems.

MATERIALS & METHODS

Cultures and sporulation

B. cereus T was obtained from our laboratory culture collection. Sporulation and harvesting was by the method of Johnson et al. (1982) on fortified nutrient agar. Harvested spores were centrifuged at 5000 × *g* for 20 min at 4 ± 2°C, washed 5 times and stored (≈10^{10} CFU/mL) in distilled water at 4°C for 15 to 20 mo.

B. stearothermophilus ATCC 12980 was obtained from American Type Culture Collection (Rockville, Maryland). The culture was sporulated using the method of Warth (1978). Harvested spores were centrifuged at 3700 × *g* for 15 min at 4°C. The spores were washed five times, stored (≈10^{8} CFU/mL) in distilled water at 4 ± 2°C for 7 mo, and then frozen at −20°C. Aliquots were thawed in the refrigerator and used within 2 wk.

Heating and recovery media

For experiments with *B. cereus*, the heating medium was 100 mM sodium phosphate buffer (pH 6.8). *B. stearothermophilus* experiments used 90 mM sodium phosphate buffer (pH 6.8). The recovery medium was 0.1-strength tryptic soy agar with 0.1% soluble starch added (TSAS) (Zechman and Pflug, 1991). This recovery medium provided improved recovery of injured spores.

Buffer and media from the same preparation were used for each matched batch and continuous run. *B. cereus* T spores were not heat shocked; to assure activation and prevent shoulders on survivor curves,

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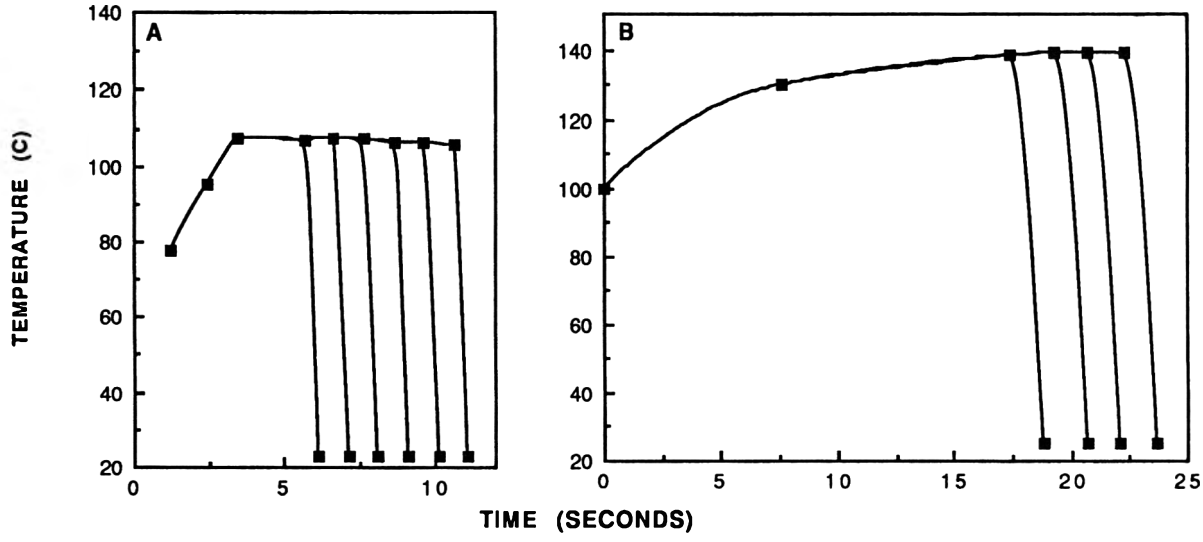

Fig. 1—(A) Typical time-temperature profile in laboratory scale thermal processing system. (B) Typical time-temperature profile in miniaturized continuous flow system.

B. stearothermophilus spores were heat shocked for 15 min at 105°C within 1 hr prior to each experiment.

Batch system for *B. cereus* T spores

A modification of the method of Stern and Proctor (1954) was used for batch heating of *B. cereus* T spores. Capillary tubes (0.8–1.1 mm i.d. × 90 mm, Kimble product number 34507, Kimble, Vineland, NJ) filled with spore suspension were heated in an oil bath (Haake D8, Paramus, NJ). Thermal inactivation kinetics were determined at 94, 99, and 103°C. Four to seven time intervals were evaluated/temperature. Each temperature was evaluated in triplicate. The temperature reported was the oil bath temperature ± range as determined by a National Institute for Standards and Technology calibrated thermometer. The capillary tubes were filled with 50 µL of spore suspension in buffer using a Hamilton syringe and the open end was sealed with an oxygen/propane flame. The capillary tubes were placed vertically into an open mesh basket. The basket was plunged into the oil bath and timing for experimental sampling began after the capillary tubes were heated for a 10 sec come-up time. This come-up time was determined in preliminary experiments to be adequate at 100 and 130°C. The heated capillary tubes (one at each sampling time) were immediately placed into freshly prepared, cold (about 0°C) hypochlorite (ca 500 ppm, pH 6.5) until the heating sequence was complete. The capillary tubes were wiped, scored, and returned to cold hypochlorite for 15 min. The ends of the capillary tubes were removed using sterile tweezers. The contents of capillary tubes were flushed from the tube with 100 µL of 0.1% peptone water. The samples were diluted in 0.1% peptone water, pour plated in duplicate, incubated at 30 to 32°C, and colonies were counted after 24 ± 2 hr. Plates were held up to 4 days to verify that injured spores were recovered within 24 hr.

Continuous flow system for *B. cereus* T spores

The laboratory scale thermal processing system (Fairchild et al., 1994) located in the NCSU Center for Aseptic Processing and Packaging pilot plant was used as the continuous flow system for *B. cereus* T spores. The system was sanitized with hypochlorite (ca. 500 ppm, pH 6.5) for 15 min and then by running hot water (>100°C) for 10 min. The system required about 15 L of inoculated product/replicate. The temperature was equilibrated to the desired experimental temperature with running water and then the system was switched from water to spore suspension after required conditions were reached. The spore suspension was pumped through heating, holding, and cooling sections of the system. The cooled waste was collected in a carboy and decontaminated (121°C, 45 min).

The pressure was maintained at 1034 kPa and the flow rate was held at 1.5 L/min. The Reynolds number at 100°C was calculated as 13,990; thus turbulent flow conditions existed. Thermocouples (Omega, Stanford, CT) in the heating, holding, and cooling sections were connected to a data acquisition system (Metrabyte Corporation, Taunton, MA) to continuously monitor temperature and time-temperature profiles recorded (Fig. 1).

Three experiments were conducted on separate days at each temperature. Samples were removed from each sample port into sterile test tubes and the computer time was noted so that temperature could be precisely extracted from the time-temperature data file. The zero-time sample was the first sample after the heating section, at the beginning of the holding tube. The samples were treated exactly as in batch experiments.

Batch system for *B. stearothermophilus* ATCC 12980 spores

The batch system for the *B. stearothermophilus* spores was identical to that used for the *B. cereus* T spores with the following exceptions: The open end of the capillary tube was sealed with a bunsen burner. Plates were incubated in plastic sleeves at 55°C; colonies were counted after 48 ± 4 hr. Plates were held for 7 days to validate that the 2 day counts included possible injured spores. Thermal inactivation experiments were performed at 128.5, 130, 133, and 135°C.

Continuous system for B. stearothermophilus ATCC 12980 spores

Because yields of *B. stearothermophilus* were low ($\approx 10^8$ spores/mL) a miniaturized continuous flow system was designed and constructed (Wescott, 1993). This consisted of a positive displacement pump (Bran-Lubbe, Buffalo Grove, IL), pump dampener (Liquid Dynamics, Hampstead, NC), 0.635 cm o.d. stainless steel tubing through the pressure gauge, and 0.318 cm o.d. coiled (ca. 5 cm diam coil) stainless steel tubing through holding and cooling tubes. Holding tubes were connected in series so that holding time was additive. The holding tubes were submerged within a hot oil bath. Valves were positioned at the end of each holding tube segment so flow could be directed to the next holding tube or through the cooling tube. The cooling tubes were submerged in an ice water bath. A back pressure valve was positioned at the end of the cooling tube. As before, thermocouples (Omega) were positioned in the center of the product flow at the end of each hold tube. The thermocouples were connected to a data acquisition system (Metrabyte Corporation, Taunton, MA) and time of sampling was noted so temperature could be precisely extracted.

As the oil bath temperature was increasing to the selected temperature, the system was sanitized with hypochlorite (ca 500 ppm, pH 6.5) for 30 min and rinsed with sterile distilled water. Hot sterile distilled water was used to equilibrate the system to selected temperatures, pressures, and flow rates prior to switching to the inoculated test suspension. The spore suspension was continuously stirred during sampling. Samples were removed in order from longest to shortest heating times since they used a common cooling coil. This was done by positioning the valve at the end of the appropriate holding tube section to divert flow through the cooling tube and collecting sample in a sterile test tube. The samples were immediately placed on ice. The zero-time sample represented product which went through the initial heating tube prior to entering the holding tube so that heat inactivation was evaluated at nearly isothermal conditions. This eliminated the need to evaluate lethality during come-up time. After completion of sampling and as the oil bath was decreasing in

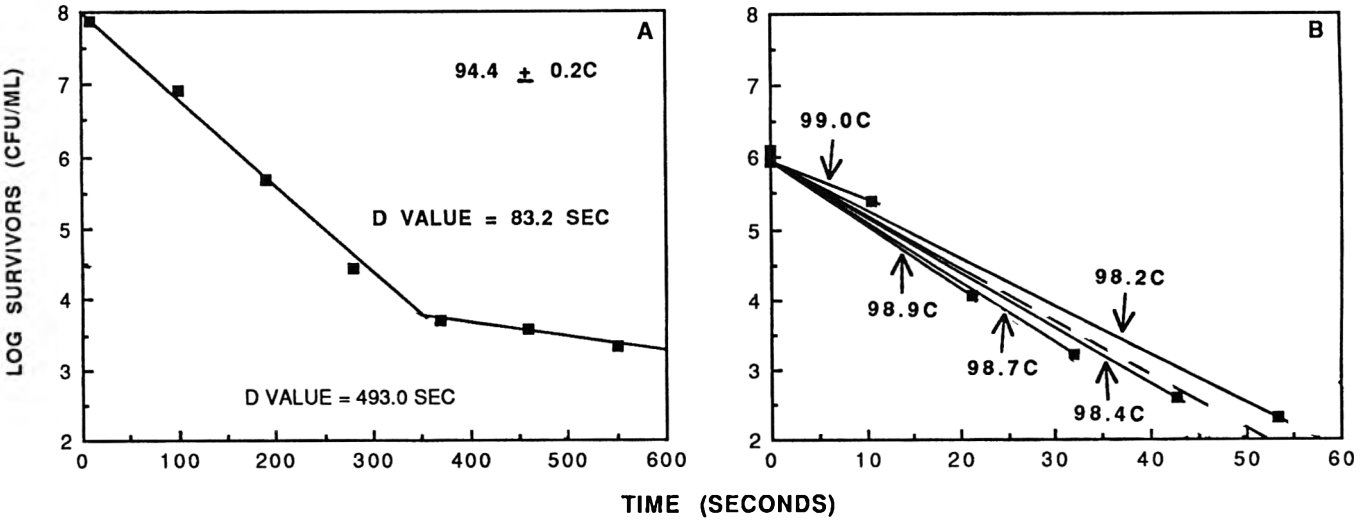

Fig. 2—Typical survivor curves for (A) *B. cereus* T spores heated in batch system where temperature was the oil bath temperature ± the range and (B) *B. cereus* T spores heated in continuous flow system [EPM (—) and traditional analysis (----)].

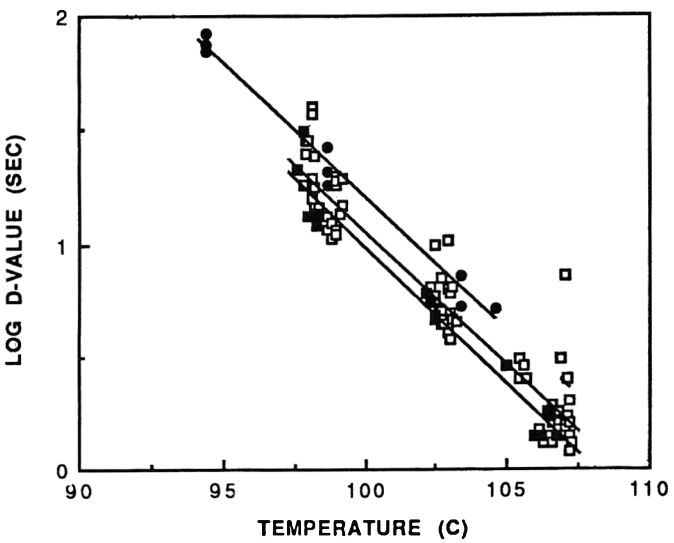

Fig. 3—Comparison of decimal reduction time curves for *B. cereus* T spores heated in batch (●) and continuous flow (■ , □) systems. Continuous flow data were analyzed by the traditional (■) and equivalent point (□) methods.

temperature, the system was sanitized with hypochlorite (ca. 500 ppm, pH 6.5) for 1 hr and then rinsed thoroughly with distilled water.

For all experiments, the flow rate was 83 mL/min. The Reynolds number at 138°C was calculated to be 1550; thus, laminar flow conditions existed, although some mixing was likely due to curvature of the heating coils. The mean residence time was determined using the method described by Hill (1977). The pressure was maintained at 1241–1276 kPa. The heat-shocked spores were added to the buffer (heated to 100°C) immediately before heating began. Time-temperature profiles for this continuous flow system were recorded (Fig. 1B).

Data analysis

At each temperature, the experiments were done in triplicate. For each replicate, the D-value (time to inactivate 90% of the population) was determined as the -slope^{-1} of the best fit line of the survivor curve ($\log_{10}$ population vs time). When tailing occurred, D-values were determined from the initial, linear portion of the survivor curves. In the continuous flow system when analyzed by the traditional method, the temperature was recorded as the average temperature through the holding tube. When analyzed by the equivalent point method, the temperature was the calculated equivalent temperature (T_E). D-values from replicate experiments were incorporated separately to construct decimal reduction time curves (log D-value vs temperature). The z_D-value (degrees of temperature change needed to bring about a tenfold reduction in D-value) was determined as the - slope^{-1} of the decimal reduction time curve. Significant differences in decimal reduction time curves were subjected to tests of homogeneity of slopes. The reaction rates, k, were determined by the equation D-value = 2.303/k. Arrhenius plots (ln k vs °K^{-1}) were prepared and activation energy, E_a, was determined. The slope of the Arrhenius plot equals $-E_a/R$ where R equals the universal gas constant (8.314 J/mol °K).

The EPM defines the thermal treatment with one equivalent time (t_E) and temperature (T_E), independent of activation energies as demonstrated by Swartzel (1984). The EPM was used to generate D-values using equivalent time, equivalent temperature, and survivor concentrations from two points. Multiple sampling times yielded multiple D-values. The initial point was the intercept of the best fit line of the survivor curve.

RESULTS & DISCUSSION

B. cereus T spore inactivation study

Typical survivor curves from the batch and continuous flow systems, were compared (Fig. 2A, B). Tailing was observed consistently in the batch system but not in the continuous flow system. We speculate that this may have been due to a minority of the population occurring as clumps of spores (about 7×10^4 spores/clump) which were disrupted by the shear forces in the continuous system (Cerf, 1077). Data from the initial linear portion of survivor curves were used to determine D-values where tails were observed. The thermal inactivation experiments for batch and continuous flow processes overlapped at 99 and 103°C. It would have been preferable to operate the two systems in the same temperature range. However, the batch system could not be used at 107°C because the sampling times were too short for accurate determination and the continuous system could not be run below 99°C because hold tubes were too long.

An activation energy of 335 kJ/mol (80.0 kcal/mol) was obtained in the batch system. Activation energies of 328 kJ/mol (78.4 kcal/mol) and 313 kJ/mol (74.8 kcal/mol) were obtained in the continuous system when analyzed by traditional and equivalent point methods, respectively. The predicted D-values (from decimal reduction time curves) at 94, 99, and 103°C were 81, 21, and 7 seconds in the batch system. These were similar to values reported for *B. cereus* spores by Johnson et al. (1982) and Rajkowski and Mikolajcik (1987). A z_D-value of 8.5°C was obtained in the batch system.

The predicted D-values at 99, 103, and 107°C in the continuous flow system were 13, 4, and 1 sec; and 15, 5, and 2 sec when analyzed by the traditional and equivalent point methods, respectively. z_D-Values of 8.2°C and 8.5°C were obtained in the continuous system when analyzed by the traditional method and equivalent point method, respectively.

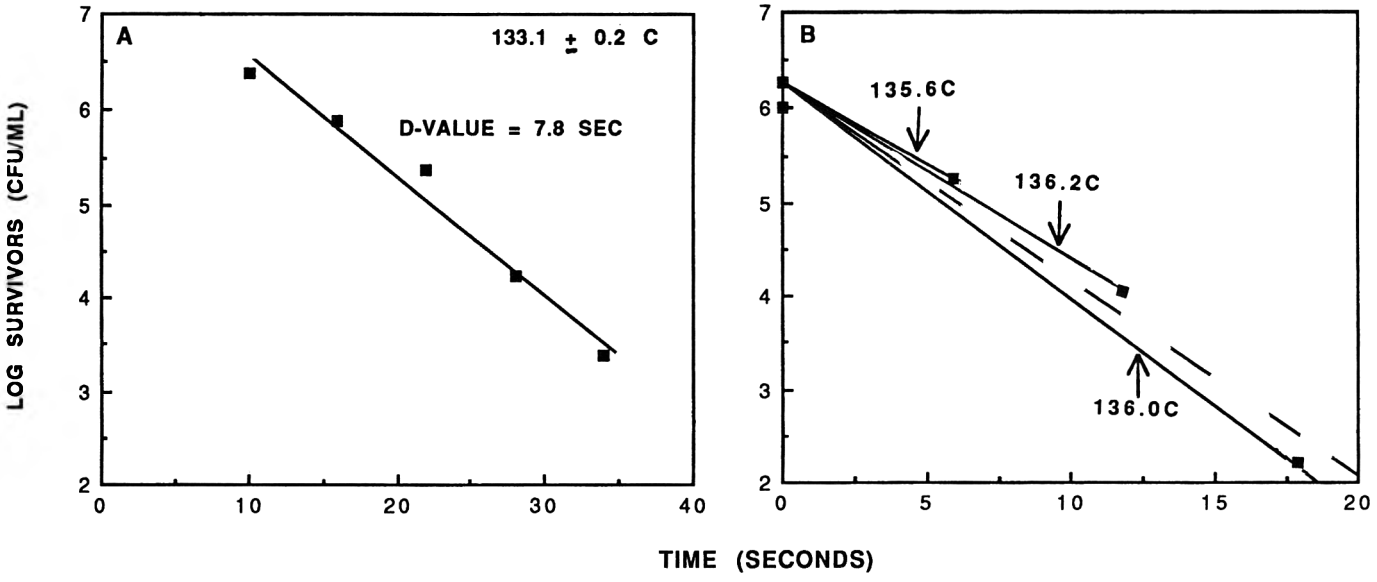

Fig. 4—Typical survivor curves for (A) *B. stearothermophilus* ATCC 12980 spores heated in batch system, where temperature was the oil bath temperature ± the range, and (B) *B. stearothermophilus* ATCC 12980 spores heated in the continuous flow system [EPM (—) and traditional analysis (---)].

Possible errors exist for both systems. While the batch method provides rapid heating and cooling, the length and volume of each sealed capillary tube were not exactly the same and there was no certainty that glass tubes had the same wall thickness (Perkin et al., 1977). Such minor differences could affect heat penetration. Other problems in the batch system included head space differences causing variation in the solution boiling point, human errors in timing, and errors due to tube position within the heating bath.

Temperature measurement was the primary possible error source with the continuous flow system. Thermocouples were positioned to monitor the center temperature of the hold tube; the temperature near the walls may be higher than at the center. Although position may have been imprecise, the Reynolds number was almost 14,000, indicating turbulent flow. The thorough mixing would reduce the temperature difference within the holding tube so that errors due to temperature measurement were probably not significant.

The decimal reduction time curves for the batch and continuous flow systems were compared (Fig. 3) as analyzed by each method. These decimal reduction time curves were parallel, as reflected by z_D-values. The intercepts of the decimal reduction time curves were significantly different, indicating predicted D-values at any temperature would be different.

The traditional Arrhenius or Bigelow models assume isothermal conditions at any given temperature. Since the continuous system was nonisothermal, applying these models introduced error. In our experiments, the temperature in the continuous flow systems dropped as much as 1.6 to 3.4°C across the holding tube. The average temperatures in the holding tubes were used in analyses. The EPM better defined the system because it can generate one equivalent time and temperature for each section across the hold tube. Thus the nonisothermal system was represented by an equivalent time and temperature which could be treated as though they represented an isothermal process. Therefore, the EPM was considered the best method for analyzing data from the continuous flow systems. Comparing the decimal reduction time curves from batch and continuous flow systems (EPM analysis) indicated the continuous system was more lethal than the batch system. Possible explanations may be the contribution due to physical forces in the continuous flow system (such as pressure and shear) which were not in the batch system. Burton et al. (1977) and Fairchild et al. (1994) suggested there may be fundamental differences in mechanisms of thermal destruction in batch and continuous flow systems. The thermal processes were defined as accurately as possible but errors in characterization of the continuous flow system may also explain the differences.

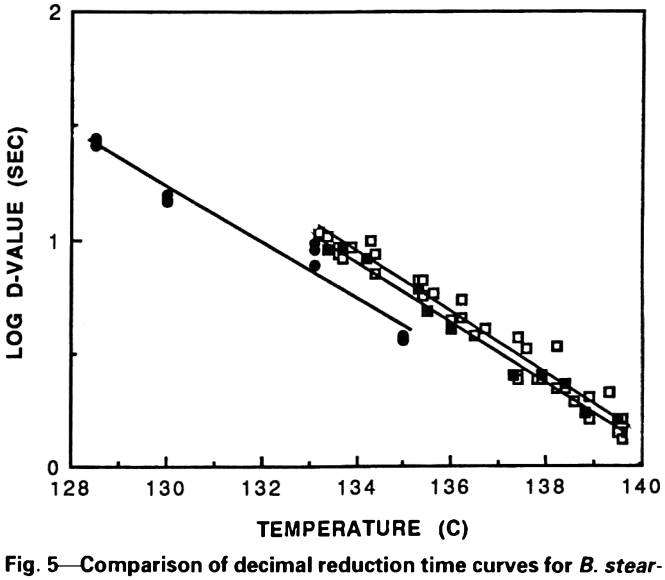

Fig. 5—Comparison of decimal reduction time curves for *B. stearothermophilus* ATCC 12980 spores heated in batch (●) and continuous flow (■ , □) systems. Continuous flow data were analyzed by the traditional (■) and equivalent point (□) methods.

B. stearothermophilus ATCC 12980 spore inactivation study

Representative survivor curves for *B. stearothermophilus* thermal inactivation in batch (Fig. 4A) and continuous (Fig. 4B) flow systems were compared. Thermal inactivation experiments for batch and continuous flow processes overlapped at 133 and 135°C. The limitation of the batch system at higher temperatures (138 and 139°C) was that short sampling times needed to achieve a minimum of a three log reduction in spore population could not accurately be obtained. The limitation of the continuous flow system at lower temperatures (below 133°C) was that the longer hold tubes, needed to achieve the necessary reduction in spore population, would create pressures too high for the system (>1310 kPa).

An activation energy of 377 kJ/mol (90.2 kcal/mol) was obtained in the batch system. Activation energies of 366 kJ/mol (87.5 kcal/mol) and 405 kJ/mol (96.8 kcal/mol) were obtained in the continuous system when analyzed by the traditional method and equivalent point method, respectively. The predicted D-values at 128.5, 130, 133, and 135°C were 26, 17, 7, and 4 seconds in the batch system. The D-values obtained were similar to published values reported by Busta (1967) and Davies et al. (1977). A z_D-value of 8.1°C was obtained in the batch system. The predicted D-values at 133, 135, 138, and 139°C in the continuous flow system were 11, 6, 2, and 2; and 12, 6, 3, and 2 seconds when analyzed by traditional and equivalent point methods, respectively. z_D-Values of 7.5°C and 7.3°C were obtained in the continuous system when analyzed by the traditional method and equivalent point method, respectively.

Possible experimental errors which may have resulted from the miniaturized continuous flow system included noninstantaneous decrease in temperature upon leaving the holding tubes and the existence of laminar flow conditions which complicated specifying temperature and time. After leaving the holding tubes, the heated spore suspension required up to 1.4 sec to reach the cooling tube. However, after leaving the holding tubes, the spore suspension immediately began to cool so that spore thermal inactivation would have been minimal. Heating time was calculated as the mean residence time and did not account for residence time distribution. This would be significant in laminar flow conditions where some particles travel faster or slower than the mean. Thermocouples were carefully positioned to monitor temperatures at the center of the hold tube. The precision of placement was critical since the temperature near the walls would be higher than at the center. Thus, the temperature reported was the minimal temperature in the system. Recognizing such sources of possible error, the continuous flow system was characterized as accurately as possible.

The *B. stearothermophilus* spore decimal reduction time curves for the batch and continuous flow systems were compared (Fig. 5) as analyzed by each method. The slope of the decimal reduction time curve for the batch system was different from the decimal reduction time curves with the continuous flow system. Data were insufficient to indicate any difference in apparent parallel slopes of the decimal reduction time curves for the continuous flow system. Comparing the decimal reduction time curves of the batch and continuous flow system (EPM analysis) indicated the batch system was more lethal.

These batch and continuous thermal systems enabled dependable documentation of the thermal exposure of the organisms and monitoring of inactivation in nearly isothermal conditions. The relative lethality of batch vs continuous flow processing, documented using *B. cereus* T spores and *B. stearothermophilus* ATCC 12980 spores, were opposite. The two obvious experimental differences were (1) the *Bacillus* species used for the thermal inactivation, and (2) the continuous flow systems which were different in flow dynamics. In an attempt to explain the reversal in relative lethality observed in the two studies, thermal inactivation of *B. cereus* T spores was conducted at 101°C in the miniaturized continuous flow system (data not shown). These results fell between the decimal reduction time curves from the previous *B. cereus* T experiments for the batch and continuous flow systems. Thus, results were inconclusive, yet indicate that both the organism and system may affect relative lethality. Further testing is needed to determine reasons for reversal of relative lethality from batch to continuous heating seen in the *B. cereus* and *B. stearothermophilus* studies. Nevertheless, our results generally agreed with Burton et al. (1977) and confirmed that using batch generated data to predict or design continuous flow processes may not be dependable.

CONCLUSIONS

WE SUGGEST LABORATORY SCALE continuous processing systems would yield the most accurate results for modeling commercial processes, especially when turbulent flow is achieved in both systems. The EPM for analysis of a biological system was successfully applied and is useful for avoiding errors due to minor temperature changes during experimentation or processing.

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Peroxidase Stability and Reactivation after Heat Treatment and Manothermosonication

PASCUAL LOPEZ and JUSTINO BURGOS

ABSTRACT

The effect of several factors on the resistance of horseradish peroxidase (POX) to heat and manothermosonication (MTS), the simultaneous application of heat and ultrasonic waves under pressure, has been studied. Reactivation after heat or MTS followed very similar patterns. Both heat and MTS inactivation of POX had a similar pH dependence. Addition of sucrose or glycerol to the treatment buffer resulted in more inactivation of POX by heat. MTS reversed these effects, especially after addition of glycerol. Addition of KCl had a slightly protective effect against both heat and MTS treatments whereas addition of glucose increased the inactivation of POX by heat and MTS. Higher concentrations of POX were less sensitive to MTS. Both heat and MTS inactivation of POX appeared to result from a common mechanism.

Key Words: peroxidase, heat, pressure, thermosonication, inactivation

INTRODUCTION

FOOD STABILIZATION requires efficient microbial and enzyme destruction or inhibition. Heat treatment, widely used to destroy microorganisms and inactivate enzymes, may have negative effects on some food properties such as color, flavor, and nutrient content or bioavailability. Interest is increasing in technological procedures to destroy or inactivate microorganisms and enzymes with little or no heat (Mertens and Knorr, 1992; Gould, 1995). One such technology proposed as alternative to heat treatments of food is manothermosonication (MTS), the simultaneous application of heat and ultrasound waves under pressure. MTS has substantially reduced microorganisms and enzymes' D values (decimal reduction times) (Lopez et al., 1994; Sala et al., 1995).

In previous studies we explored the effects of some physical parameters related to MTS efficiency, such as ultrasound amplitude or hydrostatic pressure, on peroxidase (POX), lipoxygenase (LOX), and polyphenoloxidase inactivation (Lopez et al., 1994). We also studied the effects of some chemical components and physicochemical conditions of the treatment medium on LOX resistance against MTS (Lopez and Burgos, 1995).

Our current objective was to examine the influence of several factors on the efficiency of heat and MTS inactivation of POX and its reactivation under different conditions. POX (E.C. 1.11.1.7), is a heme containing enzyme catalyzing many reactions between a peroxide and an electron donor, and has been empirically related to off-flavors and off-colors in unblanched vegetables. Because of its relatively high thermal stability it has been frequently followed to evaluate the efficiency of vegetable blanching (Williams et al., 1986).

MATERIALS & METHODS

Materials

Peroxidase type VI from horseradish was a product of Sigma (St. Louis, MO). Chemicals used were of reagent grade.

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Heat and manothermosonication treatments

Heat treatments were performed in a TR-SC thermoresistometer (Condon et al., 1989). Manothermosonication was carried out in a thermoultrasonic resistometer made by a modification of the TR-SC instrument as described by Lopez et al. (1994). The treatment chamber was equipped with an ultrasonic horn, Model 450, from Branson (Danbury, CT) which radiated ultrasonic waves at a fixed frequency of 20 kHz. MTS treatments were performed at an ultrasound amplitude of 145 μm. Pressure was applied from a nitrogen cylinder and varied between 3 and 3.5 kg/cm^2.

All treatments were performed in potassium phosphate buffers of different molarities and pH. POX (2 mg in the standard treatment) dissolved in 300 μL H_2O was injected in the treatment chamber containing 23 mL of appropriate phosphate buffer at the preset temperature (126.5°C). A thermocouple connected to a recorder measured the true temperature in the treatment chamber. Periodic removal of 100 μL samples from the treatment chamber was achieved through a solenoid valve. Samples removed were immediately cooled on ice and assayed for enzymatic activity. From the main vessel a volume of treatment medium was injected, equal to the volume of sample removed. This was taken into account for residual activity calculation.

Peroxidase assay

Peroxidase activity was determined spectrophotometrically in an Uvikon 810 spectrophotometer from Kontron (Zurich) equipped with a recorder and a thermostated cell holder block. The change in absorbance at 460 nm was measured after addition of the peroxidase containing sample (2–30 μL). The assay medium consisted of 1 mL of a 0.1M sodium phosphate buffer, pH 6.0, containing 0.01% o-dianisidine, 0.005% H_2O_2 and 2% methanol. All enzyme assays were performed in duplicate at 25°C.

Enzyme inactivation parameters

D values, (the time required to inactivate 90% of original enzyme activity), is the kinetic parameter we used to compare heat and MTS effects on inactivation of POX. D values were determined from plots of log enzyme activity vs time. Plots with correlation coefficients 0.98 were rejected. Each enzyme inactivation experiment was performed at least twice. Plots of log of residual activities vs time were used to evaluate experiments. (D/D quotients Fig. 1 and 4–6 are arithmetic means of experimental values).

Reactivation experiments

Samples (300 μL) were removed from the treatment chamber. Aliquots were immediately assayed for activity, dialyzed (150 μL), or kept at room temperature (≈23°C) or at 4°C. Dialysis was performed against the treatment buffer (20 mM phosphate buffer, pH 6.5,) at 4°C in a system 500 microdialyzer from Pierce (Rockford, IL) for 80 min using a dialysis membrane of 5000 daltons molecular weight "cut-off." After dialysis the samples were kept at 4°C and later assayed for activity.

RESULTS

pH relationship of heat and MTS peroxidase inactivation

The pH of the medium usually affects enzyme thermal resistance (Stauffer, 1989). Enzymes tend to have maximum stability at pH near an optimum pH (Webb, 1964). The effects of pH in the range 5.2–8.0 on POX inactivation at 126.5°C by simple heat treatment and MTS at 3 kg/cm^2 were compared (Fig. 1). Inactivation profiles were in all cases linear, corresponding to a

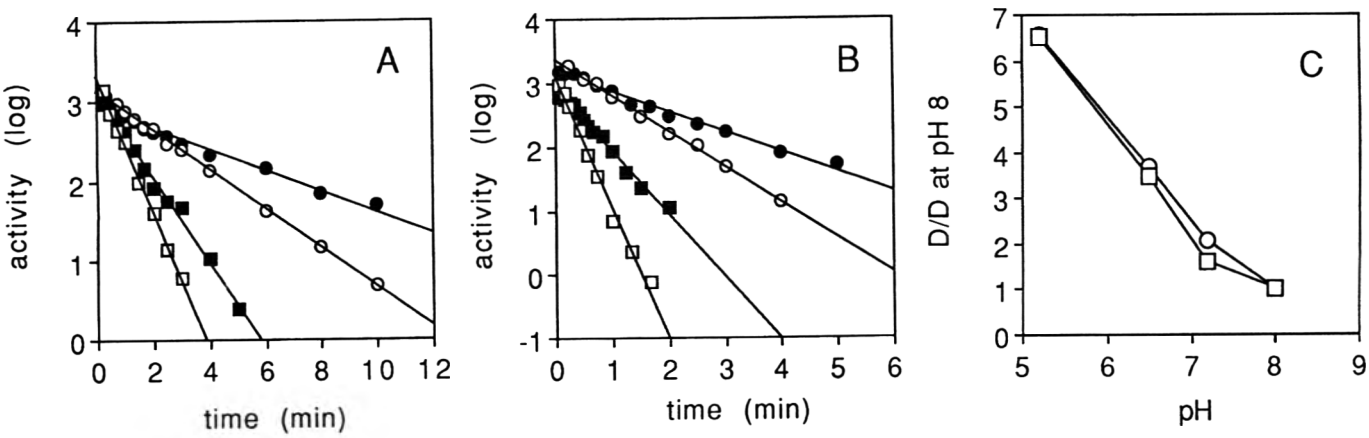

Fig. 1—Effect of pH on peroxidase resistance to heat (A) and MTS (B) at 126.5°C, 145 μm ultrasound amplitude, 20mM phosphate buffer, and 3 kg/cm^2: pH 5.2 (●); pH 6.5 (○); pH 7.2 (■), and pH 8.0 (□). (C) Change in the ratio DpH/DpH8 in MTS (○) and heat treatments (□).

first order inactivation, except for heat treatment at pH 5.2, which showed slight biphasic effects. On both treatments D increased with decreasing pH. D values were lower in MTS than in the heat treatment at any pH. However, dependence of the inactivation process on pH was the same in both treatments, (Fig. 1-C). Thus the proven synergistic effects of heat and ultrasonic waves were not affected by pH.

Effect of enzyme concentration

Protein stability against heat and other physical denaturating agents is higher in concentrated than in diluted solutions (Putman, 1953). The influence of enzyme concentration on the POX inactivating effect of both treatments was compared (Fig. 2). A slight increase occurred in MTS resistance with increasing enzyme concentration, whereas heat resistance remained unchanged. Therefore the synergistic effect of heat and ultrasound on POX destruction was slightly affected by enzyme concentration.

Effects of sugars, glycerol, and potassium chloride

Neutral salts, sugars, and polyhydric alcohols function generally as protein structure stabilizers (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982; Yoovidhya et al., 1986; Timasheff and Arakawa, 1989). Nevertheless, Chang et al. (1988) have reported a destabilizing effect of sugars on horseradish peroxidase. We therefore compared the effects of these components on the stability of the enzyme against heat and MTS.

Sucrose did not affect linearity of the plots of log residual activity vs time (Fig. 3A, B) but, increasing concentrations reduced POX heat resistance much more than its MTS resistance (Fig. 3C). Therefore, sucrose in the treatment medium decreased the effectiveness of the ultrasonic waves toward heat inactivation.

Glucose also did not affect the apparent kinetic order of the enzyme inactivation process (Fig. 4) but had an inactivating effect of similar magnitude against both treatments. It did not affect the synergism between heat and ultrasonic waves.

Glycerol produced strikingly different effects on heat and MTS resistance (Fig. 5). The heat resistance of POX diminished almost linearly with increasing concentrations of glycerol and the opposite effect appeared when POX was submitted to MTS (Fig. 5). Consequently, the effectiveness of heat and ultrasonic waves was diminished as glycerol concentration increased.

KCl at both 0.25M and 1M had a protective effect for POX against heat (Fig. 6). Against MTS this effect was similar at 1M concentration, but strikingly, POX MTS resistance decreased in the presence of 0.25M KCl.

Enzyme reactivation

POX is easily reactivated after moderate heat treatments. A comparison of reactivation after heat and MTS treatment could help elucidate the mechanism of enhanced enzyme destruction by ultrasonic waves. Reactivations at room temperature after heat and MTS treatments follow similar patterns. The less activity was reduced the higher the percentage of lost activity recovered (Fig. 7). Reactivation after both treatments was more rapid at room temperature than in the cold and did not occur after dialysis (Fig. 8).

DISCUSSION

THERMAL INACTIVATION OF POX has been widely studied for many years. POX from different sources differs substantially in thermal resistance and POX isozymes also differ in thermal stability (Kon and Whitaker, 1965; Winter, 1971; Delincée and Schafer, 1975; Williams et al., 1986; Chang et al., 1988; Robinson, 1991). A deviation of first order kinetics has been frequently observed and is generally accepted to be due to the presence in the enzyme preparation of isozymes of differing heat stabilities (Naveh et al., 1988; Chang et al., 1988). In a previous work (Lopez et al., 1994), we observed a biphasic course of thermal inactivation and first order kinetics of inactivation by MTS. The monophasic course of thermal inactivation we found here could only be attributed to greater isozyme homogeneity in the POX batch used for this study.

Our results are not in agreement with previous results on the influence of pH on POX resistance to thermal inactivation. According to Lu and Whitaker (1974), maximum stability of horseradish POX was reached at pH 7 and heat resistance diminished to about half at pH 5 and 8. Those observations were not in accordance with reports for asparagus POX; Ganthavorn et al. (1991) observed no significant differences in stability of the enzyme in the pH range 4–7.

The pH dependence of heat and MTS POX inactivation were equal. The z values, and therefore the inactivation energies, were the same for both processes (Lopez et al., 1994; Sala et al., 1994), and the renaturating behavior of heat and MTS treated samples were identical. These suggest a common inactivation mechanism. This is in contrast to results with lipoxygenase (Lopez and Burgos, 1995), where MTS and heat inactivations had different pH dependence and inactivation energy. Lipoxygenase MTS inactivation mechanisms (most likely implying free radicals arising from water sonolysis) are probably different from that for thermal inactivation.

Lu and Whitaker (1974) suggested that the mechanism in thermal inactivation of POX was the release of the heme moiety followed by denaturation of the liberated apoprotein. Our reac-

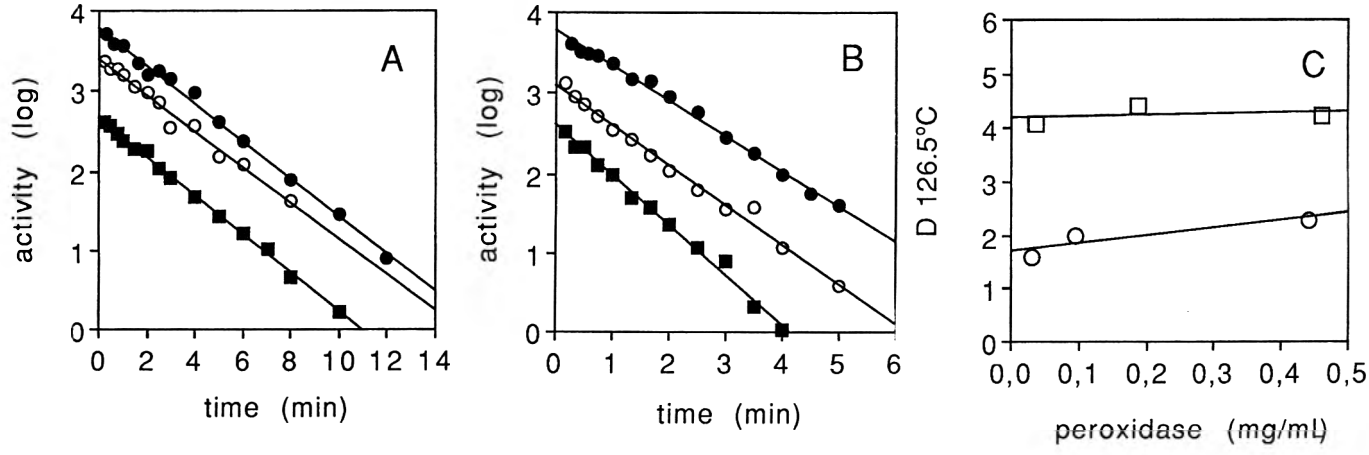

Fig. 2—Effect of POX concentration on its heat and MTS resistance at 126.5°C in 20mM phosphate buffer, pH 6.5. (A) Heat treatment of 0.04 mg/mL (■), 0.19 mg/mL (○), and 0.48 mg/mL POX (●); (B) MTS treatment (145 μm ultrasound amplitude and 3.5 kg/cm^2) of 0.03 mg/ml (■), 0.1 mg/mL (○), and 0.45 mg/mL POX (●). (C) Change in POX D value with POX concentration: (○) MTS; (□) Heat treatments.

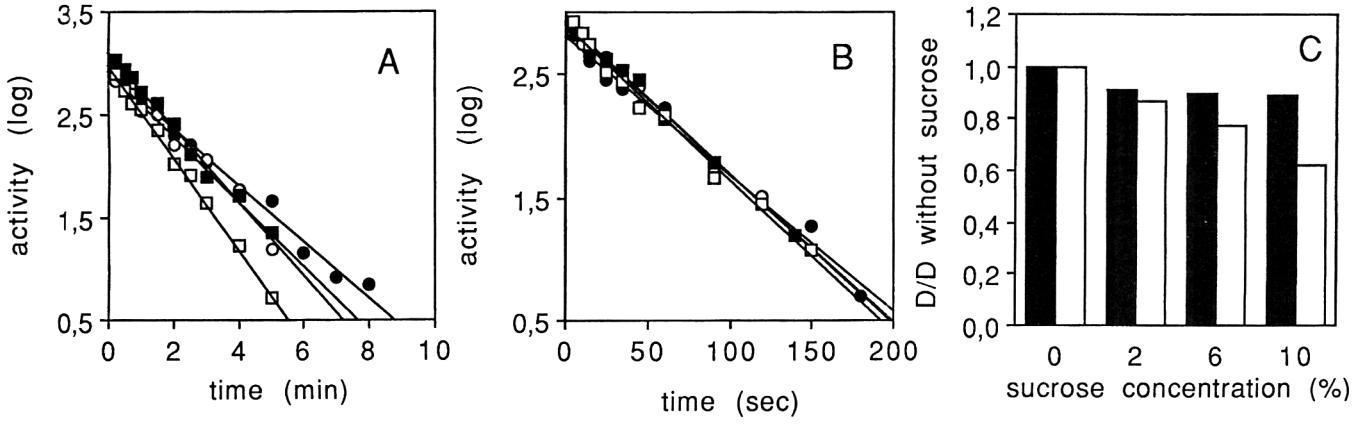

Fig. 3—Effect of sucrose concentration on POX resistance to heat (A) and MTS (B) in 40 mM phosphate buffer, pH 6.5: 0% sucrose (●); 2% sucrose (○); 6% sucrose (■), and 10% sucrose (□). Treatment conditions as in Fig. 2. (C) Ratio D value in the presence of sucrose/D value without sucrose in heat (white bars) and MTS (black bars) treatments.

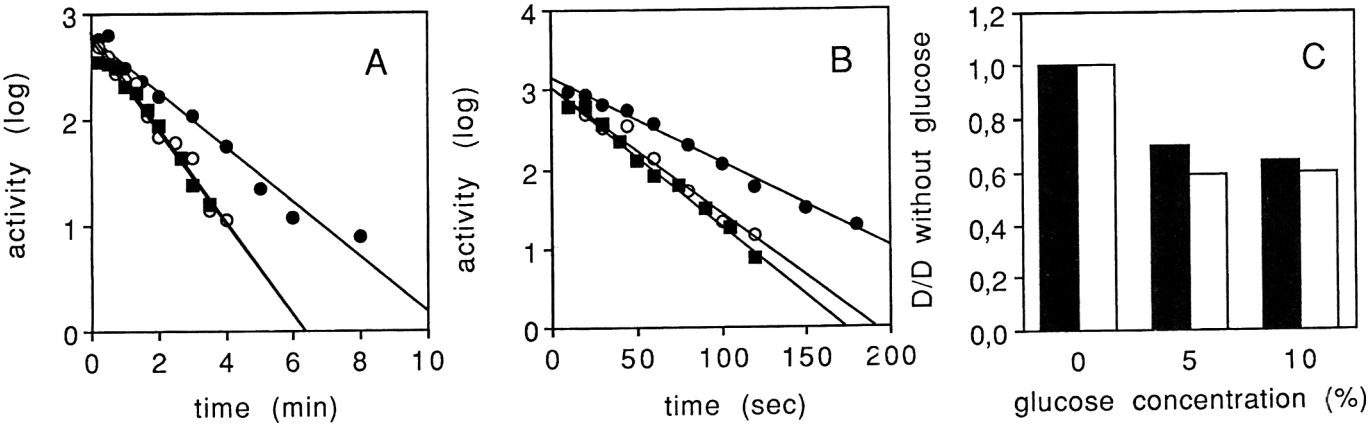

Fig. 4—Effect of glucose concentration on POX resistance to heat (A) and MTS (B) in 40 mM phosphate buffer, pH 6.5: 0% glucose (●); 5% glucose (○), and 10% (■) glucose. Treatment conditions as in Fig. 2. (C) Ratio D value in the presence of glucose/D value without glucose in heat (white bars) and MTS (black bars) treatments.

tivation experiments support such mechanism for both thermal and MTS inactivation, since dialysis after partial inactivation by any of them diminished its reactivation. Note that a different mechanism implying secondary attack of the enzyme by free radicals produced by radiolysis has been proposed for POX inactivation by gamma irradiation (Macris and Markakis, 1971; Metodiewa et al., 1987). The free radicals involved are hypothesized to be hydroxy, peroxy and superoxide. Free radical formation by decomposition of water inside the oscillating bubbles during ultrasound irradiation is well established (El'Piner et al., 1965). However, our experimental results were not compatible with a similar mechanism in POX inactivation by MTS. The

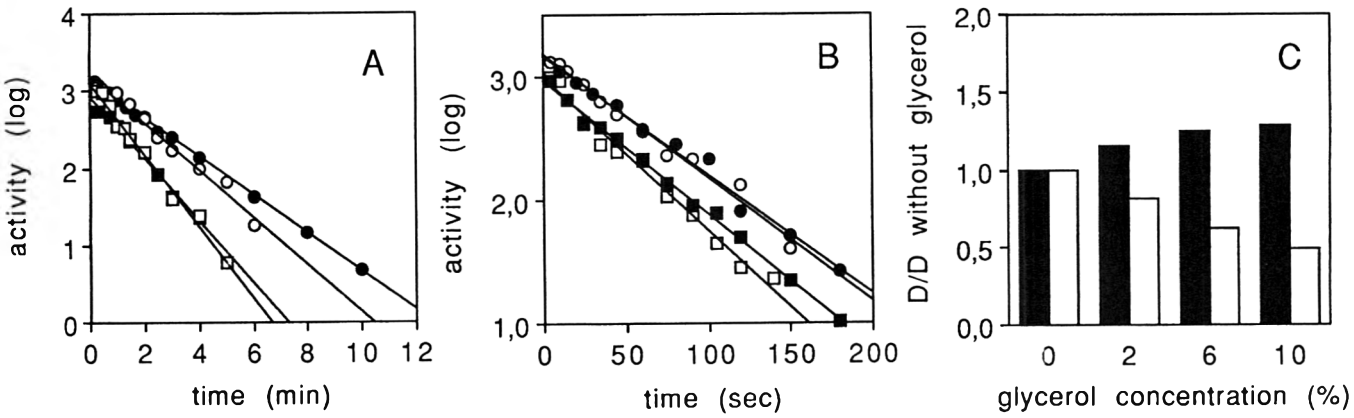

Fig. 5—Effect of glycerol concentration on POX resistance to heat (A) and MTS (B) in 20 mM phosphate buffer, pH 6.5: 0% glycerol (●); 2% glycerol (○); 6% glycerol (■), and 10% glycerol (□). Treatment conditions as in Fig. 2. (C) Ratio D value in the presence of glycerol/D value without glycerol in heat (white bars) and MTS (black bars) treatments.

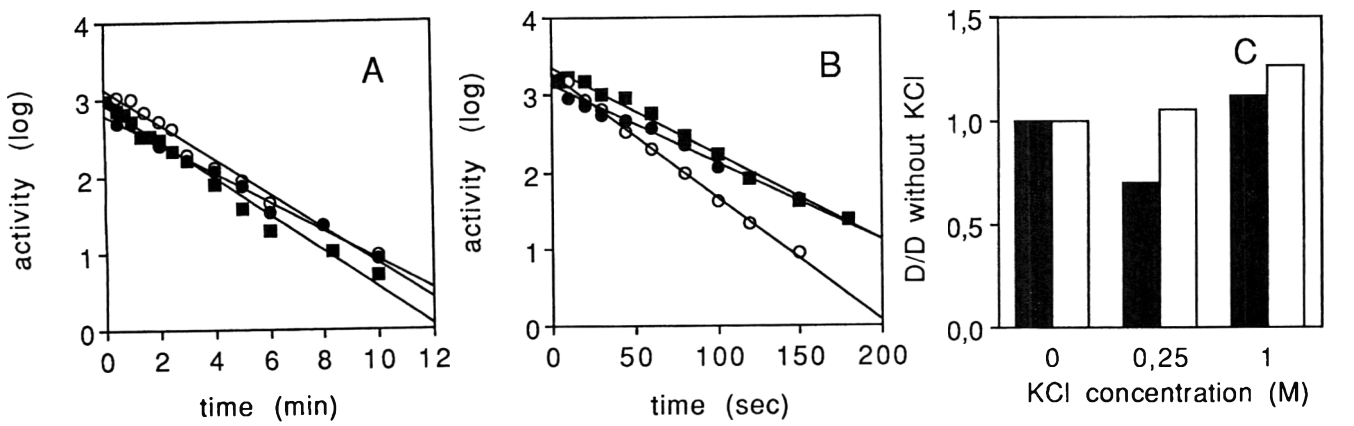

Fig. 6—Effect of KCl concentration on POX resistance to heat (A) and MTS (B) in 40mM phosphate buffer, pH 6.5: OM KCl (■); 0.25M KCl (○), and 1M KCl (●). Treatment conditions as in Fig. 2. (C) Ratio D value in the presence of KCl/D value without KCl in heat (white bars) and MTS (black bars) treatments.

only effect which may support such mechanism would be the slight protection produced by increasing enzyme concentrations.

The higher POX inactivation effectiveness of MTS vs heat in all systems could be due either to enhanced heme dissociation or apoenzyme denaturation. Weissler (1960) has proved that ultrasonic radiation of hemoglobin splits heme from globin. Thus enhanced heme dissociation seems probably most important in the POX inactivating efficiency increased by MTS over that of heat alone.

The influence of pH on thermal stability reported by Lu and Whitaker (1974) has been attributed to the fact that release of hemin from enzyme is pH dependent (Maehly, 1952, 1953), occurring most rapidly below pH 5. In our enzyme preparation the influence of pH on hemin release may have been slight as suggested by inactivation rates reported by Lu and Whitaker (1974), in the pH range 5.2–8.0. Also, this influence may have been obscured by the pH effects on other steps of inactivation such as apoenzyme denaturation.

Lu and Whitaker (1974) reported a great decrease of POX thermostability in the presence of NaCl with eightfold increase in initial rate of inactivation in the presence of 0.6M NaCl. Our experiments with KCl showed, on the contrary, a slight protection of the enzyme against both heat and MTS treatments with increasing salt concentration. This would be in accordance with hypotheses on cosolute protection of protein structure by preferential exclusion (Timasheff and Arakawa, 1989; Timasheff, 1993). According to such theory the preferential exclusion of inert solutes from the macromolecule surroundings should protect the native structure because their binding would increase the free energy of the system. This unfavorable position would be increased in the denatured state, because of the increase in solvent surface contact. Thus the equilibrium between the native and denatured states would be directed by these cosolutes toward the native conformation.

The influence of 1M KCl on POX inactivation under heat and MTS is very similar to that previously observed on LOX. In LOX, 1M KCl had a small heat protective effect which was lost when ultrasonic waves were simultaneously applied (Lopez and Burgos, 1995). This could be attributed to the distortion of the native enzyme molecules by the ultrasonic waves to the limits of their flexibility. This would decrease the differences in surface areas between the native and denatured states. This would, in turn, diminish differences between the increases of free energy induced by KCl when it bound to the native protein or when it bound to the denatured form. We could not explain the fact that 0.25M KCl decreased POX MTS resistance.

Sugars and polyhydric alcohols have been reported to protect the native conformation of proteins against thermal denaturation (Back et al., 1979; Lee and Timasheff, 1981; Kella and Poola, 1985; Graber and Combes, 1989). However Chang et al. (1988) showed that sucrose, glucose, fructose, and lactose (at 10%) accelerated POX thermal inactivation. Sucrose was the least effective of the sugars we tested in reducing thermostability. The efficiency of the other sugars increased with their reducing power. Thus the effect was attributed to the interaction of the reducing groups with the protein amino acids. Our results were in agreement with those of Chang et al. (1988) only inasmuch as they showed that POX thermostability was reduced by su-

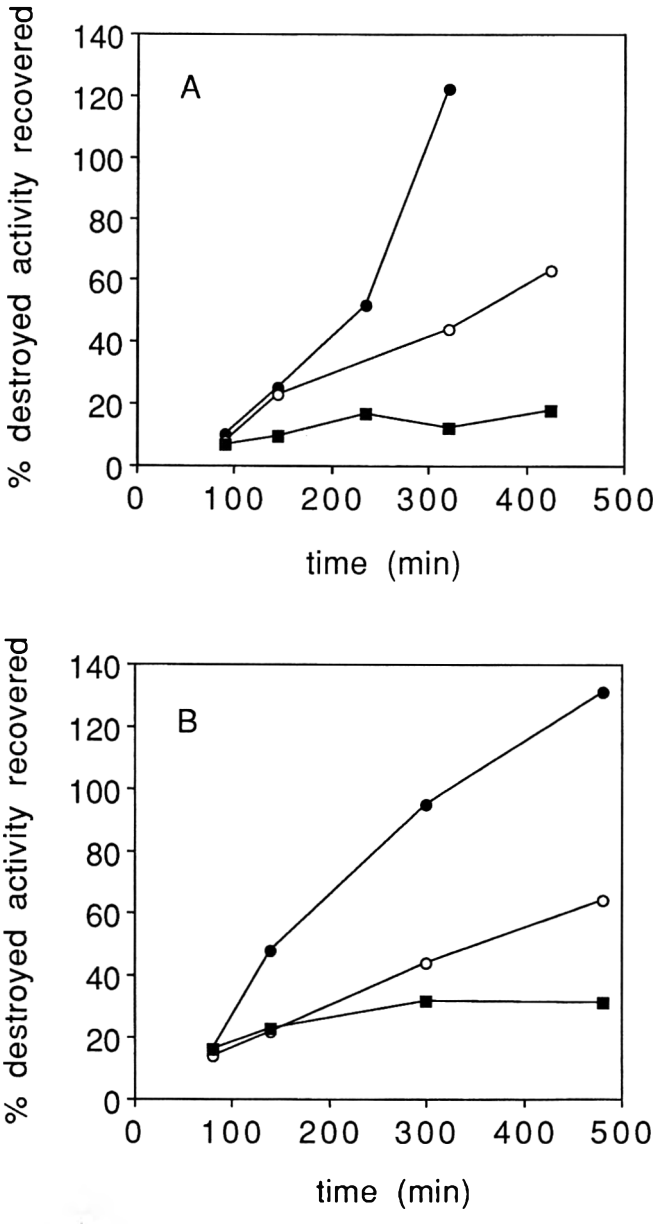

Fig. 7—Dependence of activity recovery at room temperature from degree of inactivation. (A) Reactivation of peroxidase samples after heat inactivation up to 14% (●), 42% (○), and 69% (■). (B) Reactivation of peroxidase samples after MTS inactivation up to 24% (●), 36% (○) and, 62% (■).

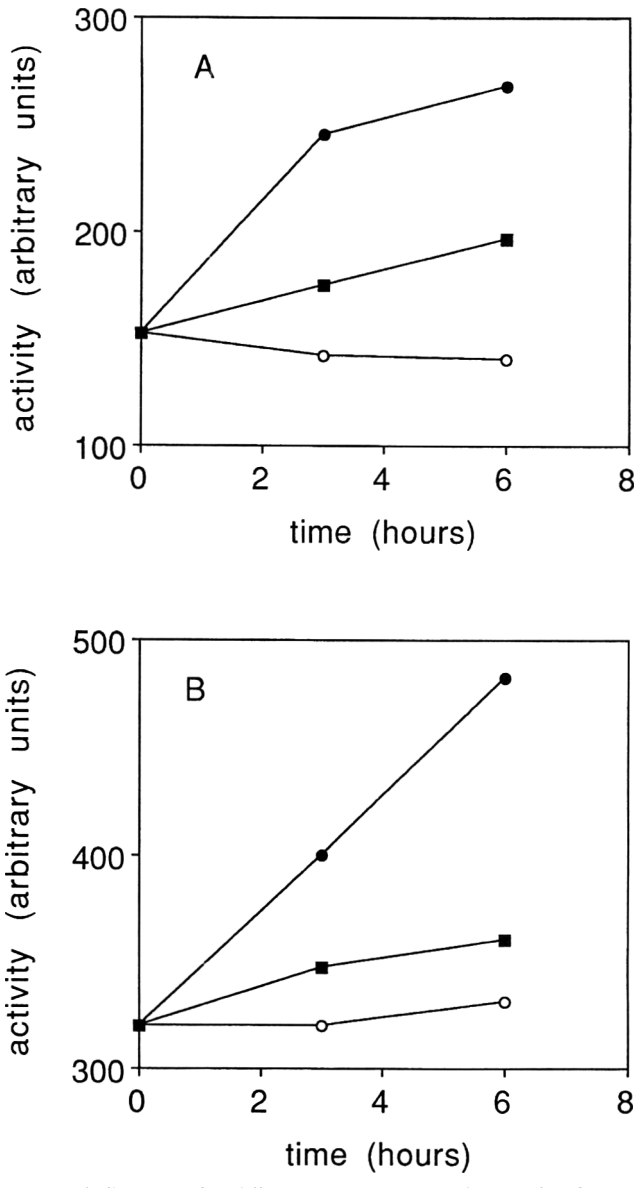

Fig. 8—Influence of holding temperature and dialysis after heat (A) and MTS (B) treatments on peroxidase reactivation: samples kept at room temperature (●); samples kept at 4°C (■); samples dialyzed and kept at 4°C (○).

crose and glucose. The degree of thermostability reduction we observed was extremely different from that previously published. From our results, glucose and sucrose were about equally efficient. Both decreased the D value of thermal inactivation by a factor of about 1.7. In the results of Chang et al. (1988) the D value was decreased by glucose by a factor near 30 and by sucrose by a factor <1.5. At the higher treatment temperature of our experiments, sucrose may have undergone some degree of inversion and the fructose and glucose so generated could be responsible for the inactivation. However, glycerol, a polyhydric alcohol devoid of reducing power, caused similar inactivation at the same concentration. This indicates a more general effect unrelated to reducing groups. The destabilizing effect of glucose and sucrose was reduced and that of glycerol reversed by MTS (although we expected a decrease in MTS efficiency due to the increase in viscosity of the medium). These results could be compatible with a mechanism in which an increase of protein-solvent surface contact becomes thermodynamically more favorable. This is what happens with denaturing cosolvents. Nevertheless glycerol as well as sucrose and glucose are preferentially excluded by nonspecific mechanisms (Timasheff, 1993). This type preferential exclusion results always in protein structure stabilization. Chang et al. (1988) proved by scanning differential calorimetry that sucrose, glucose, and fructose protected POX against heat denaturation. It seems more plausible therefore that sugars and glycerol accelerate POX inactivation by inducing a shift of the equilibrium:

POX → heme − apoenzyme, toward the right.

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Probe Length and Filling Material Effects on Thermal Conductivity Determined by a Maximum Slope Data Reduction Method

NIKOLAOS VOUDOURIS and KAN-ICHI HAYAKAWA

ABSTRACT

The influence of probe length to diameter ratio (λ) and filling material on the thermal conductivity (k) probe technique was studied using glycerol with probes of four different λs (4.1, 7.5, 13.6, and 54.3) filled with either high k paste or air by applying a maximum slope data reduction method. Statistical analysis showed the highly significant influence of λ and significant influence of the filling material. Noncalibrated k values of the smallest λ probes were 93% above the published value, whereas those of the largest λ probes were virtually identical to the published value. Separate measurements on chocolate pudding and starch gels showed that 7.5 λ k were virtually identical to the largest λ probe k when the former probes had been calibrated using glycerol. The use of short air-filled probes is not recommended when the maximum slope method is applied.

Key Words: thermal conductivity probe, filling material, heater wire length

INTRODUCTION

LINE HEAT SOURCE PROBES have been widely used to determine thermal conductivities of food because of short response time, low cost, and simplicity. All data reduction methods for this determination, are based on an approximate analytical solution of the heat conduction equation for a line heat source (Carslaw and Jaeger, 1959). Briefly stated, heat is generated at a constant rate along an infinitely long line heat source of no mass and no volume placed in an infinite medium of uniform initial temperature. At sufficiently long times, the relationship of the temperature rise at a close neighborhood to the source vs the natural logarithm of time becomes linear and thermal conductivity (k) is determined from the slope of the temperature data reduction curve:

$$k = (Q/4\pi)/[dT/d(\ln t)] \quad (1)$$

where Q is the heat input/unit length/unit time. Since the thermophysical structure of a real probe deviates from assumed conditions of the line heat source analytical solution, many researchers have investigated the influence of such structural deviation on k determination to optimize probe design and operation (Murakami et al., 1993a, Murakami et al., 1993b).

Two important probe design factors are the filling material inside the probe (Karwe and Tong, 1992) and the probe heater wire length (van der Held and van Drumen, 1949; Healy et al., 1976). Different fluid materials have been tested to fill the internal void space. D'Eustachio and Schreiner (1952) found that an air space within the probe ≤50% the probe diameter did not affect k determination of insulating materials when sufficiently low heat inputs were used. Morley (1966) used an epoxy resin as filler for application in muscles, fats and bones. There were no significant differences between k values of glycerin and agar gels determined using oil-filled and air-filled probes (Sweat, 1986). Tong and Lund (1989) used mercury as filler for frozen food applications to increase the rate of heat transfer inside the probe. Silicon rubber was used to fix the wires in the probe tube and prevent electrical contact (van Loon et al., 1989). Simulation study by Karwe and Tong (1991) showed that use of different filling materials, e.g., air, mercury and a high conductivity paste (Omegatherm 201), significantly influenced k determination. Gratzek and Toledo (1993) used a silicone oil-filled probe with an uninsulated thermocouple junction for fast response for solid food applications at high temperatures. The high thermal conductivity paste was also used by Wang and Hayakawa (1993b) to minimize temperature drop inside the probe. Murakami et al. (1993a) discussed the effect of filling material on the thermal mass of the probe and on the calibration values. They mentioned that thermal stability was an important consideration for selecting proper filling materials.

The most important departure from the line heat source theoretical analysis stems from the finite heater wire length. The heater wire of a sufficient length is required to approximate the infinitely long line heat source. e.g. no axial heat conduction, radial conduction only. However, there is need for miniaturizing the k probe for measuring thermal conductivity of small food samples. Blackwell (1956) derived an analytical expression for finding the upper limit of the axial flow error as a function of the probe length/diameter, and probe and sample thermophysical properties (one of the assumptions being homogeneous probe generating heat uniformly throughout probe volume). A length/diameter (λ) of 25 was suitable for accurate determination of k. Sweat and Haugh (1974) reported no significant differences between mean k values obtained by probes of 25.4 to 44.5 mm length ($31 < \lambda < 55$). They measured k of 19 mm long food samples using a probe with λ of 47 (38.1 mm length × 0.81 mm diameter). They noted that either probes of smaller λ values could be used for smaller sample applications or samples of shorter axial dimensions could be tested without reducing the λ of the probes. According to Murakami et al. (1993b), the proper λ should be based on an acceptable axial flow error for the intended application using Blackwell's theoretical analysis. Representative probe length/diameter and probe dimensions published for various food applications were compared at a descending order of λ values (Table 1). Note that all tabulated λ were >25, the recommended ratio by Blackwell.

An initial curvilinearity occurs in a temperature data reduction curve due to the thermophysical deviation of a probe from the assumed conditions of the line heat source theory. The linear part of the curve was identified by either a time correction factor method (van der Held and van Drunen, 1949), a maximum r^2 method (Murakami and Okos, 1988), or a maximum slope method. Wang and Hayakawa (1993b) examined theoretically and experimentally the applicability and validity of the method. One advantage of the maximum slope method (MSM) is that no probe calibration using a reference material of known k (reference k should be close to test sample k) is required for k determination. The maximum slope of the temperature data reduction curve is the closest approximation to the line heat source solution. They reported that k values estimated by the MSM were comparably accurate and less statistically variable compared to those determined by the other two widely used data

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Table 1—Representative thermal conductivity probe length/diameter (λ) and probe dimensions published for food applications at a descending λ order

λ	Length (mm)	Diameter (mm)	Application	Source
80	102	1.27	Meats and Fats	Morley (1966)
80	254	3.17	Potato Salad	Dickerson and Read (1968)
63	80	1.27	Starch Gels	Wang and Hayakawa (1993a)
59	39	0.66	Food	Sweat (1986)
55	32	0.58	Dairy and margarine	Sweat and Parmelee (1978)
48	305	6.35	Agricultural and Food	Monhensin (1980)
48	61	1.27	Carrots and Potatoes	Gratzek and Toledo (1993)
47	38	0.80	Potato	Wang and Brennan (1992)
38	19	0.50[a]	Fruits and Vegetables	Sweat (1974)
32	40	1.25	Granular Starch	Drouzas and Saravakos (1988)
32	22.8	0.71	Prototype[b]	Murakami et al. (1993b)
31	25.4	0.81	Small Samples	Sweat and Haugh (1974)
31	56	1.83	Frozen Material	Tong and Lund (1989)
30	38	1.25	Seafood Powders	Rahman et al. (1991)

[a] Constructed by wrapping thermocouple wire outside the probe tubing
[b] Based on Sweat's design (1974, 1986)

reduction methods with ideal probe calibration (e.g. k of test sample = k of calibration material). No work has been reported on the influence of filling material and heater wire length on k determination using the MSM. Moreover, there is a need for examining the probe response for λ less than 30 and studying possible interactions of filling material with heater wire length. Our objective was to study such relationships on thermal conductivity by the MSM.

MATERIALS & METHODS

A PROPER PROBE DESIGN would minimize inherent sources of error in the thermal conductivity probe method, which would be more important for probes of smaller dimensions. Figure 1 shows the cross-section of probes we used. This resulted after testing probes of design alternatives. The key probe section, i.e. the part exposed to a test sample, consisted of: stainless steel tubing, constantan heater wire, thermistor bead and lead wires, and filling material. Note that the length of this section was identical to the heater wire length. This ensured accurate estimation of heat transmitted from the probe to surrounding test materials. The heat source was a Teflon insulated (0.076 mm PFA insulation) constantan wire (0.076 mm diam). Probes with four different key section lengths, 80, 20, 11, 6 mm ($4.1 < \lambda < 54.3$), filled with either air or a high thermal conductivity paste (OT-201, Omega Engineering, Inc., Stamford, CT) were fabricated. Several probes for each filler and length combination were constructed to test the reproducability of the probes and to expedite experimental procedures. Probes exhibiting significantly different or abnormal response were eliminated by performing paired t-tests. Data obtained from 2 probes of each design combination were used. One additional probe of 6 mm length was constructed containing a silicone lacquer (Thermcoat SL, Omega Engineering) in the key probe section because a shorter probe would be more sensitive to the various design parameters. The temperature sensor was an interchangeable miniature thermistor (Mitsubishi International Corp., Tokyo) and its lead wires were also Teflon insulated (0.127 mm PFA insulation). Both Constantan and thermistor wires were placed in a 1.473 mm o.d. stainless steel tubing (K-HTX-17TW, Small Parts Inc., Miami, FL) in loop fashion, with the thermistor bead located at the midpoint of the heater wire. The tubing was sealed at the tip with an epoxy putty (Propoxy20, Hercules Chemical Co., Inc., NY). Teflon insulated copper wires were used as heater lead wires (0.076 mm dia., 0.076 mm PFA insulation). A fine welding of constantan and copper wires was achieved by adjusting the power supply of a welder so that the two formed beads were as small as possible to fit in the stainless steel tubing. By this means, any error in evaluating power dissipated per unit length of heater due to a heater length segment remaining in the end connectors would be minimized. The two formed beads were insulated with silicone lacquer (Thermcoat SL). The copper and thermistor wires were threaded through a support tubing and soldered to miniature connectors (Omega Engineering). Initially, a heat shrinkable (1.524 mm minimum expanded i.d.) Teflon support tubing (K-SMT-18, Small Parts) was used, but after preliminary probe testing, this was replaced by a teflon rod (Small Parts) with a hole drilled in it (3.175 mm o.d., 1.524 mm i.d.) to increase the strength and durability of the probe. The Teflon and stainless steel tubing were cemented together with an epoxy adhesive (Duralco 4538, Cotronics Co., Brooklyn, NY). The insertion length of the stainless steel tubing into the

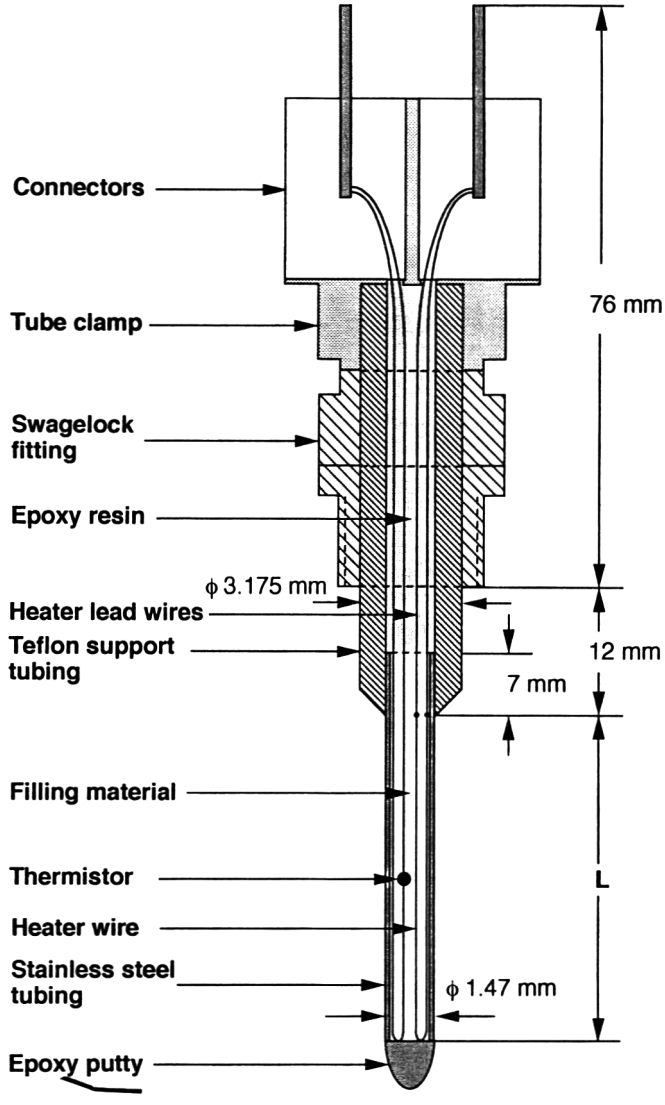

Fig. 1—Cross section of thermal conductivity probe.

teflon support did not influence k measurement. However, the shorter the insertion length the greater tendency for probe breakage. The insertion length was about 7 mm (Fig. 1) for all fabricated probes as used by Gratzek and Toledo (1993). Epoxy resin (Easypoxy, Conap, Inc., Olean, NY) was used to seal the top of the stainless steel tubing and fill the void space of the support tubing. The support tubing was fitted on the upper lid of a sample holder with a Teflon Swagelock connector (T-200-1-2, R.S. Crum & Co., Mountainside, NJ). The epoxy resin, Teflon support tubing, and Teflon Swagelock connector were used to minimize the axial heat flow losses from the probe to its surroundings. The support tubing and swagelock fitting were attached to miniature connectors with a dual element tube clamp (DX-BRLK-1/8"-SMP, Omega Engineering). For probes of 80 mm in heater wire lengths, a sample holder made of aluminum alloy (Wang and Hayakawa, 1993a) was used to perform k measurements. For the 20, 11, and 6 mm lengths, a stainless steel sample holder (28 mm i.d., 26 mm height) with minor design modifications was made based on a published theoretical analysis for the minimum sample size (Vos, 1955).

The effect of filling material and heater wire (probe) length on the thermal conductivity probe method was tested experimentally using glycerol (99.5%) as the test sample. Additional experiments were performed using both the 11 and 80 mm long probes with chocolate pudding and high-amylose corn starch (Hylon 7) gels of 3 and 4 kg water/kg dry solids to validate glycerol results. The chocolate pudding was acquired from a local retail store, and the starch gels were prepared as described by Maroulis et al. (1991).

Each sample was filled into a sample holder and experimental measurement began after the sample was equilibrated at room temperature (23–24°C). Power input levels of 5.5 to 11.7 W/m were used, resulting in temperature rises of 7–15°C. The temperature-time data were collected

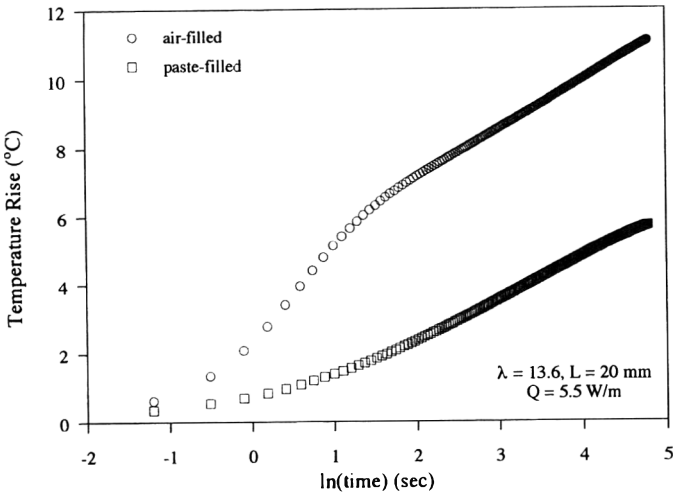

Fig. 2—Experimental temperature histories of air-filled and paste-filled 20 mm heater wire length probes for glycerol, 24°C initial temperature, I = 0.16 A.

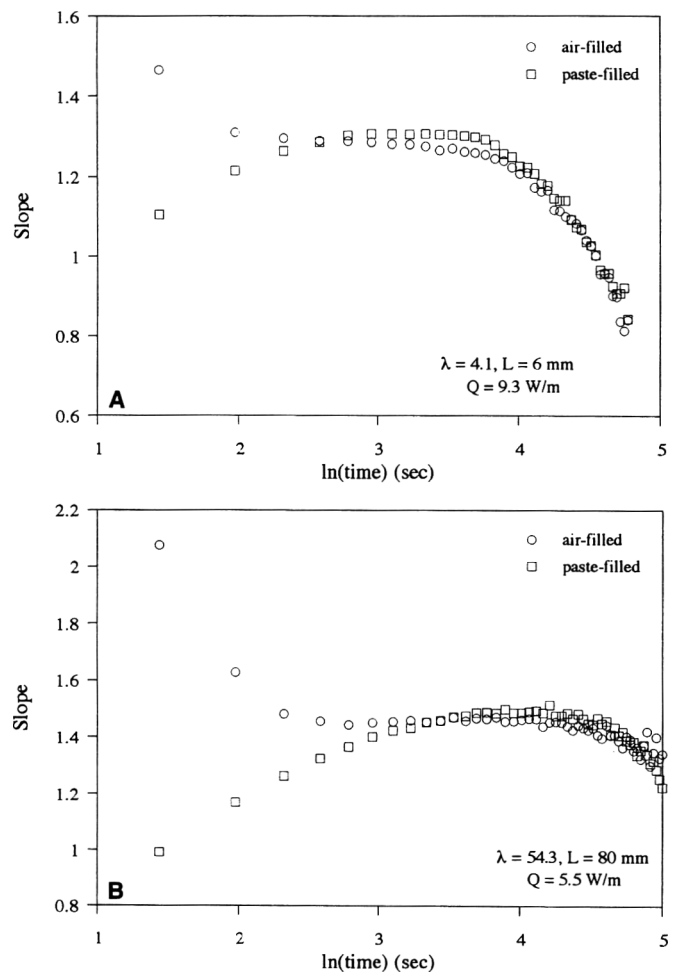

Fig. 3—Typical local slope values of paste-filled and air-filled probes obtained through successive linear regression analyses of experimental temperature-time data for glycerol, 24°C initial temperature. (a) L = 6 mm. (b) L = 80 mm.

for about 100–140 sec at 0.3 sec intervals with a computer based data acquisition system as described by Wang and Hayakawa (1993). The maximum slope data reduction method was applied to the temperature response curve (temperature rise vs natural logarithm of time) by estimating the local slopes, and consequently local k values (Eq. 1), through successive linear regression analyses. Ten data points were used to estimate one slope value. The calculated k corresponded to the mean k value in the stationary part of the experimental curve at the average stationary temperature. Twelve k replicate determinations of glycerol were made for each fabricated probe.

RESULTS & DISCUSSION

TYPICAL EXPERIMENTAL TEMPERATURE HISTORIES were compared (Fig. 2) for air-filled and paste-filled 20 mm (λ = 13.6) heater wire length probes for the same heat inputs (5.5 W/m). Similar responses were obtained from probes of other lengths. As expected, both histories had an initial nonlinear region, a time lag followed by an approximately linear region. The temperature response curve of the air-filled probe reached the linear region much faster than the paste-filled probe resulting in a much higher temperature rise inside the air-filled probe. The initial deviation from linearity and its length as well as the shifting of the temperature response curve could be attributed to the difference in thermal mass ratio of sample to probe between the air-filled and paste filled probes (Murakami et al., 1993a). The heat capacity of paste, k = 2.38 W/(mC°), ρ = 2380 kg/m^3, C = 3830 J/(KgC°), is much larger than that of air, k = 0.026 W/(mC°), ρ = 1.165 kg/m^3, C = 1172 J/(KgC°) (Karwe and Tong, 1991). No differences in slope of the linear region between probes with different filling material of same lengths were visually observed.

Typical local slope values were compared for paste-filled and air-filled probes obtained through successive linear regression analyses of experimental temperature-time data for 6 (λ = 4.1)(Fig. 3a) and 80 mm (λ = 54.3) (Fig. 3b) long probes. The applied heat inputs were 9.3 and 5.5 W/m respectively. The local slopes of paste-filled probes increased initially, reached a stationary region of maximum value and then, for later times, decreased as reported (Wang and Hayakawa, 1993b). However, for air-filled probes, the slope values decreased before also reaching a stationary part of the 'maximum slope' curve. This was probably due to the differences in the initial nonlinear region of the temperature response curves of paste-filled and air-filled probes (Fig. 2). Therefore, the stationary slope rather than the 'maximum slope' value was used for k determination with air-filled probes. Visually (Fig. 2), the local slopes of the linear region of the temperature-time plots for paste-filled and air-filled probes appeared visually to be identical but slope values were slightly lower for air-filled probes (Fig. 3a and 3b). This was in agreement with results of a theoretical simulation study reported by Murakami et al. (1993a). As the thermal mass of the probe decreased, the calculated k increased. The maximum slope (stationary slope for air-filled probes) was reached sooner with the 6 mm than with the 80 mm probes whether paste-filled or air-filled. Shorter the length or smaller the λ of the probe, the greater the axial end effects and the larger the axial flow error. Since the maximum slope, i.e. minimum k, was observed just prior to the occurrence of axial effects (Asher et al., 1986), it would be detected at shorter times when the λ was smaller. Note that the stationary part of the slope-ln(t) plot was narrower for shorter probes (about 20 sec for a 6 mm probe and 50 sec for an 80 mm probe). Furthermore, short air-filled probes had a disadvantage compared to paste-filled ones. A true stationary part in the slope-ln(t) plot of the 6 mm long air-filled probe was not observed, but the mean k was determined from a quasi-stationary part (Fig. 3a). This may lead to inaccurate k determination. Therefore, short air-filled probes are not recommended when the maximum slope data reduction method is to be used.

The calculated k values were subjected to a two-way analysis of variance to examine the statistical significance of the filling material (air and paste) and the heater wire length on k determination (Anonymous, 1991). Results showed that the heater wire length and filling material significantly influenced k, whereas significant interaction of heater wire length occurred with the filling material (Table 2).

Table 2—Two-way analysis of variance of the effect of filling material and heating wire length on glycerol thermal conductivity[a]

Source of variation	DF	Sum of squares	F Ratio	Level of significance
Filling Material	1	0.0002827	4.6651	0.0335 *
Length	3	0.9452273	5199.348	0.0000 *
Filing Material × Length	3	0.0002744	1.5093	0.2177
Error	88	0.0053327		
Total	95	0.9511172		

[a] The asterisk signify the parameter, which influenced significantly thermal conductivity.

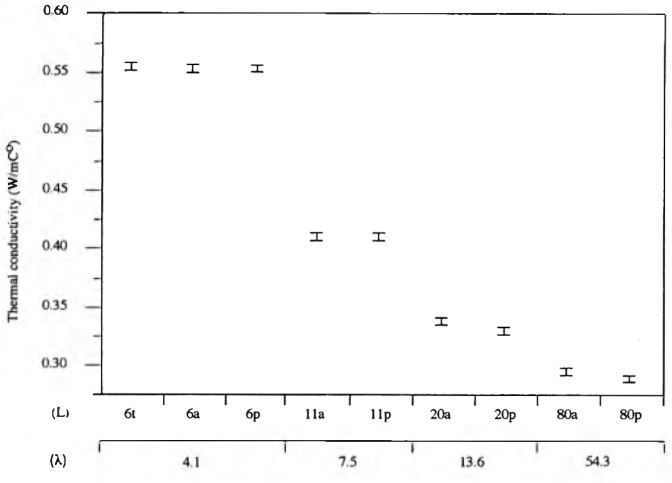

Fig. 4—Analysis of variance diagram for comparing 95% confidence intervals of thermal conductivity means of 12 k values of glycerol for probe design groups with different heater wire lengths, 6, 11, 20, 80 mm, filled with either p = paste or a = air or t = Thermcoat SL (30°C).

For detailed examination of these results, the 95% confidence interval for the mean value of each design group was estimated (Fig. 4). Mean k values of design groups of different lengths were significantly different (Table 3) confirming the strong influence of the heater wire length. Groups demonstrating no significant different mean k values, were all pair combinations of filling material (silicone lacquer, air, or paste) for the 6 mm length as well as the 11 mm air-filled with the 11 mm paste-filled design group. However, for 20 and 80 mm lengths, the means of air-filled compared to paste-filled probe designs were significantly different. Mean k values of short probes were larger than those of long probes and mean k's of air-filled probes were slightly larger compared to those of paste-filled ones. Noncalibrated k values determined using the 6 mm long probe ($\lambda = 4.1$) deviated about 93% from both the published and the 80 mm ($\lambda = 54.3$) probe mean k values. The differences between mean k values of probes with identical length and different filling material ranged from 2.5% for 20 mm long probes (1.75% for 80 mm) to ≈0% for 6 and 11 mm probes. Standard deviation values were larger for paste-filled probes than air-filled ones. Standard deviations decreased overall with decreasing heater wire length. This was because the local slope curve of the shorter probe had the well defined local slope value. A shift in slope increase to decrease occurred within a narrower time span compared to the local slope curve of the longer probe (Fig. 3a and 3b). Therefore, the maximum slope value may be determined more accurately with the shorter probe. However, there were greater probe-to-probe variations with shorter probes compared to longer probes because of difficulty in making the shorter ones. This did not influence results since probes with dissimilar or abnormal responses were eliminated.

Noncalibrated k values of glycerol determined by paste-filled and air-filled probes were compared (Fig. 5) for different λ ratios and regression curves were obtained Eq. (2) and (3):

Table 3—Calculated k values of glycerol at 30°C for different probe designs and percent difference from published values

Length (mm)	λ (–)	Filler	Mean (W/mC°)	Std Dev (W/mC°)	Std Dev of mean[a] (W/mC°)	% Difference[b] (–)
6	4.1	silicone lacquer	0.5544	0.0024	0.0007	92.8
6	4.1	air	0.5528	0.0126	0.0036	92.2
6	4.1	paste	0.5527	0.0160	0.0046	92.2
11	7.5	air	0.4110	0.0021	0.0006	42.9
11	7.5	paste	0.4106	0.0024	0.0007	42.7
20	13.6	air	0.3378	0.0013	0.0004	17.5
20	13.6	paste	0.3296	0.0025	0.0007	14.6
80	54.3	air	0.2950	0.0031	0.0009	2.6
80	54.3	paste	0.2899	0.0047	0.0013	0.8

[a] For 12 replicate determinations
[b] (calculated k-published k)/published k × 100

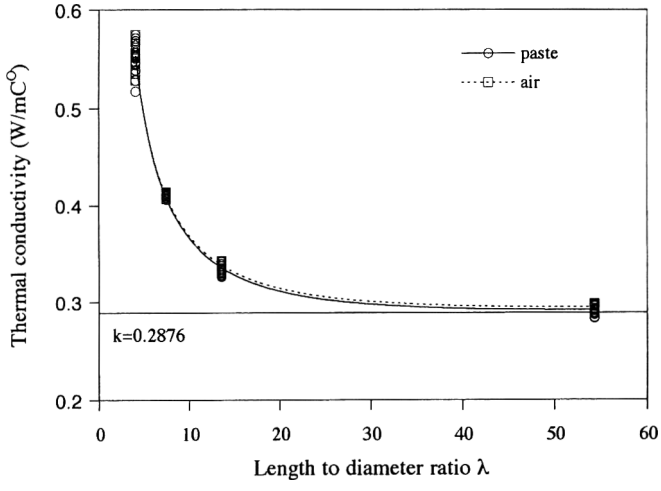

Fig. 5—Influence of length/diameter on experimental thermal conductivity values of glycerol determined by air-filled and paste-filled (30°C).

Paste-filled

$$k = 1.4187\ \lambda^{-1} + 0.17836\ \lambda^{0.099989}, \qquad r^2 = 0.99673 \quad (2)$$

Air-filled

$$k = 1.3951\ \lambda^{-1} + 0.18435\ \lambda^{0.094726}, \qquad r^2 = 0.99767 \quad (3)$$

The smaller the λ, the larger the axial flow error and the larger the determined k (Blackwell, 1956). Starting with the lowest λ value, estimated k decreased sharply with increasing λ, then the decrease became more gradual, and ultimately, a stationary region was reached ($\lambda > 30$) at the neighborhood of the published value of 0.2876 W/(mC°) (Incropera and De Witt, 1991). This was in agreement with studies by Wang and Hayakawa (1993b) for sufficiently long probes. They used a paste-filled probe with λ equal to 63 and reported that the MSM did not require probe calibration. In our work, for λ equal to 54.3, the differences between determined thermal conductivities and published values were almost zero for paste-filled probes (<1%) and 2.6% for air-filled ones. The initial steep decrease of the curve indicated that a probe of very small λ was more sensitive to design and operating changes, such as heater wire length, filling material, time interval for regression analysis, as well as positioning of the temperature sensor inside the probe. The larger the λ, the closer the approximation to the ideal theoretical analysis of an infinite-line heat source and the less the potential sources of error. Thus, high precision during fabrication is required (accurate wire length, precise positioning of sensor) to provide reproducible results with short probes.

Thermal conductivity may be determined accurately using a probe with a small λ when a calibration factor (published k/

Table 4—Noncalibrated and calibrated thermal conductivity values of chocolate pudding and starch gels and standard deviations for paste-filled probes of different heating wire lengths at 30°C

	Length = 11 mm						Length = 80 mm			
Sample	N[a]	k (W/mC°)	Std dev (W/mC°)	C.F.[b]	Cal. k (W/mC°)	Std dev (W/mC°)	N	k (W/mC°)	Std dev (W/mC°)	Literature value
Choc. pudding	4	0.6685	0.0031	0.7037	0.4704	0.0022	4	0.4766	0.0088	0.535[c]
High-amylose gel										
3.0[d]	2	0.7418	0.0144	0.7037	0.5220	0.0101	2	0.5261	0.0020	0.515[e]
4.0[d]	3	0.7498	0.0012	0.6999	0.5248	0.0008	2	0.5510	0.0071	0.523[e]

[a] Number of replicates, a minimum of four measurements per replicate.
[b] Calibration factor.
[c] From Sweat and Parmelee (1978).
[d] Water content, kg/kg d.b.
[e] From Maroulis et al. (1991).

calculated k) is introduced. Experimentally determined k values and their standard deviations for chocolate pudding and high-amylose starch gels containing 3 and 4 kg water/kg dry solids using paste-filled probes of 11 (λ = 7.5) and 80 mm (λ = 54.3) heater wire lengths were compared (Table 4) with their published values. Thermal conductivity values determined by 11 mm probes were calibrated using glycerol as reference material to account for axial flow error, whereas values determined by the 80 mm probes required no calibration. The difference between 11 and 80 mm probe k's of chocolate pudding was < 1.5%. Both k values were > 10% smaller than the published value because the moisture content of chocolate pudding we used was 67.5% compared to the 72.4% (published data) chocolate pudding. With the starch gels of the two moisture levels, the 11 mm probe k's were near the published values (<1.4% difference) and to the 80 mm probe k's (<4.7% difference). Therefore, a short probe of 11 mm may be used to determine sample k's using the MSM when the probe is calibrated with glycerol.

CONCLUSIONS

THE PROBE LENGTH diameter ratio λ had a significant effect on accuracy of the thermal conductivity probe method because of axial flow error. The effect of filling material, e.g. the thermal mass of the probe, was small and may be effaced when smaller probe diameters are used. The selection of a probe with small diameter was limited to the available thermistor's diameter. Short paste-filled or air-filled probes (λ < 30) may be used for accurate k determination of food when they are calibrated. Probe calibration could be accomplished by using suitable reference materials or a sufficiently long probe (λ > 30) and the maximum slope data reduction method. However, very short air-filled probes would not produce reliable k data since they do not present a true stationary part in the slope-ln(t) plot. Therefore, probes filled with high conductivity materials are strongly recommended.

NOMENCLATURE

k	Thermal conductivity, W/(mC°)
C	Specific heat, J/(kgK)
C.F.	Calibration factor
D	Probe diameter, mm
L	Probe length, mm
N	Number of replicates
Q	Power input, W/m
T	Temperature, °C
t	Time, sec
λ	Probe length to diameter ratio
ρ	Density, kg/m^3

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Microbiological, Chemical, and Sensory Changes in Irradiated *Pico De Gallo*

L.R. HOWARD, G.H. MILLER, JR., and A.B. WAGNER

ABSTRACT

The effects of gamma processing (1 kGy) and refrigerated storage (2°C) on microbiological, sensory, and chemical quality of *pico de gallo* was studied. Color, flavor, texture, odor, and heat sensory attributes were not affected by radiation treatment. The treatment decreased populations of aerobic mesophilic, heterofermentative, and total lactic microflora during storage. L-ascorbic acid content declined 50% in response to gamma processing, but levels were similar in irradiated and nonirradiated samples after 6 wk. Pectin solubility was affected by radiation treatment. Gamma processing caused a reduction in pectin degree of esterification, and conversion of chelator soluble to dilute alkali soluble and nonextractable pectins.

Key Words: *pico de gallo*, irradiation, minimally processed, pectin, ascorbic acid

INTRODUCTION

MEXICAN STYLE FOODS include *pico de gallo*, a cold salad prepared by chopping and mixing fresh tomatoes, onions and jalapeño peppers. Since the product is fresh and not pasteurized, it requires refrigeration. Exposing *pico de gallo* to low levels of gamma radiation may reduce microbial populations and extend refrigerated shelf life.

Many researchers have reported gamma irradiation as a potential treatment for extending the postharvest life of fresh fruits and vegetables (Kader, 1986; Urbain, 1986; Thomas, 1988). However, irradiation with dosages to accomplish intended purposes has resulted in softening of many horticultural food crops (Maxie et al., 1971; Kader, 1986; Urbain, 1986). Several studies indicated that such softening was associated with changes in pectic substances (McArdle and Nehemias, 1956; Kertesz et al., 1956; Somogyi and Romani, 1964, Howard and Buescher, 1989).

Gamma processing may be used to control growth of spoilage causing microorganisms and increase shelf life of fresh horticultural crops. Tomatoes exposed to > 1 kilogray (kGy) showed a reduction in decay and increased shelf life (Bramlage and Lipton, 1965; Abdel-Kader et al., 1968a), but fruit maturation influenced the effectiveness of irradiation. Pink or ripe fruit had greater shelf-life increases from radiation treatment than less mature fruit (Abdel-Kader et al., 1968a). Mold growth on fresh peppers was also reduced when fruit were irradiated at 1 to 3 kGy (Farkas et al., 1966).

Ascorbic acid is sensitive to radiation exposure but losses have rarely exceeded 20–30% (Thomas, 1988). Sensitivity of ascorbic acid to irradiation varies according to commodity, genotype, and dosage. Maxie and Summers (1968) reported a 8.6% loss of ascorbic acid in tomatoes exposed to 4 kGy, while ascorbic acid content in strawberries exposed to 2 kGy was unaffected by irradiation treatment. Also, irradiation reduced ascorbic acid more in pink and red tomatoes than in mature green tomatoes (Abdel-Kader et al., 1968b).

Minimally processed refrigerated (MPR) fruits and vegetables have limited shelf life due to spoilage microorganisms and physiological changes induced by wounding. Exposure of MPR products to low levels of irradiation (< 1 kGy) may reduce spoilage microorganisms resulting in extended shelf life with minimal changes in chemical and sensory quality. Our objective was to determine the microbiological, sensory and chemical quality of *pico de gallo* as affected by gamma processing and storage at 2°C.

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MATERIALS & METHODS

Processing and storage

Fresh tomatoes, yellow onions, and jalapeño peppers were purchased from a local supermarket. Ingredients were diced using a Hallde RG-7 food processor (Paxton Corp., Shelton, CT). Diced samples were dipped in a 100 µg/mL chlorine bath to reduce populations of surface microorganisms and blotted dry prior to packaging. Each batch of *pico de gallo* contained; 1453g tomatoes, 1067g yellow onions, 227g jalapeño peppers, and 27.2g NaCl. Mixed product (340g) was placed into ≈475 mL high density polyethylene containers and stored at 2°C. The containers had an oxygen transmission rate of 2325 cc/m²/24 hr and water transmission rate of 5.2 g/m²/24 hr.

Samples were irradiated at Steri-Genics (Ft. Worth, TX) with Co^{60} for 2–3 min to receive a dose of 1 kGy. Irradiation was conducted at room temperature. Radiation dosages were confirmed by film dosimetry, and were ± 10% of calculated doses. Control samples received no irradiation treatment. Sampling was conducted after 0, 1, 2, 3, 4, 5, and 6 wk storage at 2°C.

Microbiological analysis

Samples (10g) from each container were placed in sterile bags containing 90 mL of sterile 0.1% peptone diluent (Difco) and pummeled for 1 min in a Stomacher-400 (Tekmar Company, Cincinnati, OH). Plate counts were determined by placing 1 mL (divided over four plates) of the sample homogenate and then 0.1 mL of sample homogenate and appropriate tenfold dilutions of the same on prepoured, dried agar plates. Next, samples were evenly spread on the surface of the plates with a sterile, bent, glass rod. Three different types of agar were used: Tryptic Soy Agar (TSA), Lactobacilli MRS broth containing 1.5% agar, and APT agar. Plates were incubated for 48 hr at 25°C in air before counting and reporting the plate count/g for each sample.

Sensory analysis

Color, texture, odor, and heat were evaluated by a trained 10-member sensory panel. Evaluation was carried out under fluorescent light at room temperature (25 ± 1°C). Panelists were served 50 mL samples in three-digit randomly coded containers. They were instructed to use a plastic teaspoon to evaluate samples. Samples were served in random order and all samples were expectorated followed by rinsing with deionized water. A structured 10-point scale was used with 0 = none, 10 = intense.

Chemical analyses

L-Ascorbic acid. *Pico de gallo* (20g) was homogenized in 20 mL of 3% citric acid and filtered through Whatman 541 filter paper. Filter cake was re-extracted with 50 mL of 3% citric acid, refiltered, and the two filtrates were combined. A C_{18} Sep Pak cartridge was used to remove interfering compounds prior to HPLC analysis. C_{18} cartridges were preconditioned by washing with 4 mL methanol followed by 10 mL of deionized water. *Pico de gallo* extract (4 mL) was passed through the C_{18} cartridge. The first 3 mL were discarded and next 1 mL was collected for HPLC analysis. Samples were filtered through 0.45 µm filters and injected into a Spectra Physics Model 2000 HPLC equipped with a 100

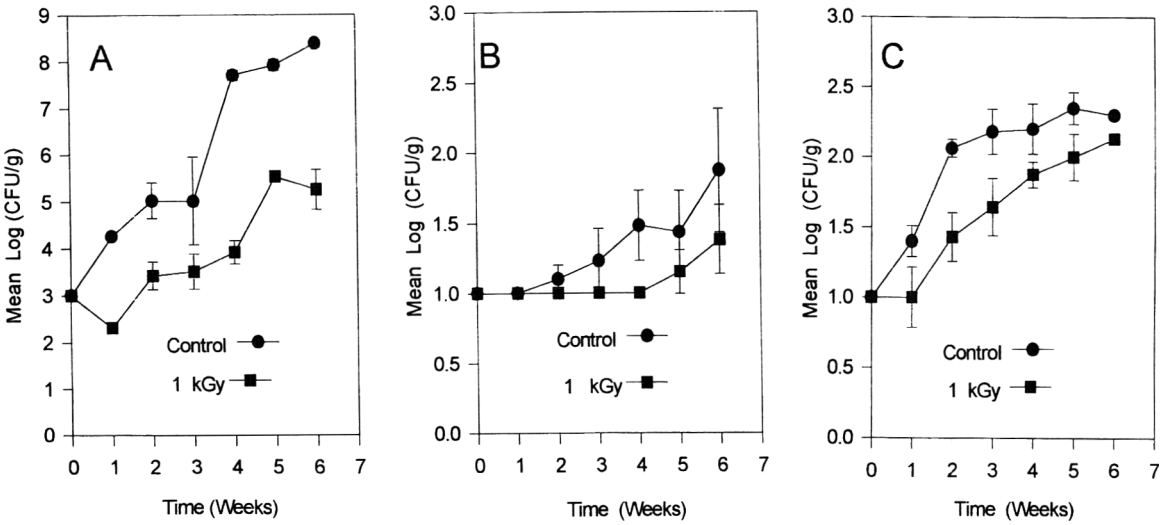

Fig. 1—Changes in populations of (A) aerobic mesophilic, (B) heterofermentative lactic, and (C) total lactic microflora in *pico de gallo* as affected by gamma processing (1 kGy) and storage at 2°C. Bars represent standard error of the mean.

× 7.8 mm HPAH Fast Acid column (Bio-Rad, Hercules, CA). The mobile phase was 0.005M H_2SO_4 at 0.4 mL/min. L-ascorbic acid was detected at 254 nm using a Spectra Physics Model 100 variable wavelength detector, and quantified using external standards.

Package headspace composition. Concentrations of oxygen, carbon dioxide and nitrogen were analyzed by thermal conductivity (TCD) and ethanol content by flame ionization (FID) gas chromatography. Ethanol and headspace gas concentrations were quantified using external standards.

Pectin composition. Cell walls were extracted from tomato pericarp tissue (100g) by boiling in 5 volumes (wt/vol) of 95% ethanol for 10 min and filtering through miracloth (CalBiochem). The residue was twice resuspended in 75% ethanol, blended and filtered. Next samples were rinsed three times (100 mL) with a 1:1 mixture of chloroform and methanol. This was followed by three 100 mL rinses with petroleum ether and three 100 mL rinses of acetone. The final alcohol insoluble solids (AIS) were dried under vacuum at 50°C and ground to a fine powder.

Total pectic substances and those extractable in water (WSP), hexametaphosphate (CSP), dilute sodium hydroxide (OHSP), and nonextractable pectins (NXP) were extracted and assayed as previously described (Howard and Buescher, 1989). Aliquots of each fraction were assayed for uronic acids by the m-hydroxydiphenyl method of Kinter and Van Buren (1982). Methoxyl content was determined using the GC method described by McFeeters and Armstrong (1984).

Statistical analyses

Data represent the mean of four replications with each container of *pico de gallo* serving as a replication. Data were analyzed by analysis of variance (SAS Institute, Inc., 1985), and mean separation was conducted using Duncan's multiple range test ($P < 0.05$).

RESULTS & DISCUSSION

GAMMA PROCESSING at 1 kGy effectively reduced microbial populations in *pico de gallo* during storage (Fig. 1). TSA agar was a general purpose media used to culture a wide variety of aerobic bacteria, while APT and lactobacilli MRS were used to culture heterofermentative and total lactobacilli, respectively. Irradiation initially reduced the population of aerobic mesophilic microflora 2-log^{10}. After week one, aerobic mesophilic microflora grew at similar rates in irradiated and non-irradiated samples, but irradiated samples maintained lower counts throughout the study. At week six mesophilic microflora in all non-irradiated samples were too numerous to count, estimated at $>2.5 \times 10^8$. In contrast, irradiated samples had TSA counts $<1 \times 10^6$. Populations of aerobic mesophilic microflora were also reduced in shrink-wrapped sweet corn irradiated at 1 kGy (Deak et al., 1987), and packaged shredded carrots irradiated at 2 kGy (Chervin and Boisseau, 1994). The bacteriostatic nature of gamma irradiation was also observed with heterofermentative and total lactic microflora. Irradiation treatment resulted in reduced counts on MRS and APT agars, but differences between non-irradiated and irradiated samples were small compared with those observed on TSA agar. Irradiation treatment greatly suppressed the growth of total lactobacilli (MRS) up to 4 wk storage. Low populations of lactobacilli in irradiated and non-irradiated samples may be attributed to the gas atmosphere in the container. A slight modified atmosphere was established during storage, with oxygen levels of 14–18% and carbon dioxide 3.5–5.6%. Lactobacilli typically proliferate under much greater carbon dioxide levels (Brackett, 1987).

Sensory properties of *pico de gallo* were unaffected by irradiation treatment ($P > 0.05$, data not shown). Effect of gamma irradiation on sensory properties varied according to dose, exposure time and food composition (Urbain, 1986). Apparently, the composition of *pico de gallo* helped resist off odor and flavor development when irradiated at 1 kGy. High levels of endogenous antioxidants and/or low lipid content may have helped retard oxidative reactions and off flavor development. Onions, jalapeño peppers and tomatoes are good sources for natural antioxidants (Russell, 1986; Leighton et al. 1994; Howard et al., 1994). The large initial decline in tomato L-ascorbic acid content indicated that antioxidants were consumed during gamma processing. The initial L-ascorbic acid concentration in diced tomatoes, 20.6 mg/100g was similar to values reported for fresh tomatoes (13.1–18.8 mg/100g; Russell, 1986). Gamma processing reduced the L-ascorbic acid content by >50%, from day 0 to day 7 (Fig. 2). However, after 5 wk storage the difference in ascorbic acid content between irradiated and non-irradiated samples was negligible. These results differed from those obtained with sweet potatoes irradiated at a dose of 1.5 kGy in which ascorbic acid declined 0.8 mg/100g after processing. Apparently, the effect of minimal processing, slicing and dicing created an environment more susceptible to oxidative damage. However, irradiation treatment possibly resulted in conversion of L-ascorbic acid to dehydroascorbic acid.

Oxygen and carbon dioxide levels in containers were not affected by gamma processing. Oxygen levels were >13% and carbon dioxide levels <5.6% in all containers. Factors affecting modified atmospheres include product respiration rate, container oxygen transmission rate, container volume and storage temperature (Kader et al., 1989). The high oxygen levels were unexpected since wounding typically increases plant respiration rates (Varoquaux and Wiley, 1994), and the containers used had low oxygen transmission rates. Perhaps a hermetic seal was not obtained, which would have enabled more oxygen to diffuse in

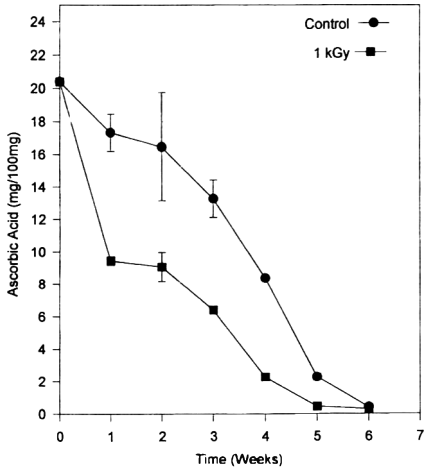

Fig. 2—Changes in concentration of ascorbic acid in *pico de gallo* as affected by gamma processing (1 kGy) and storage at 2°C. Bars represent standard error of the mean.

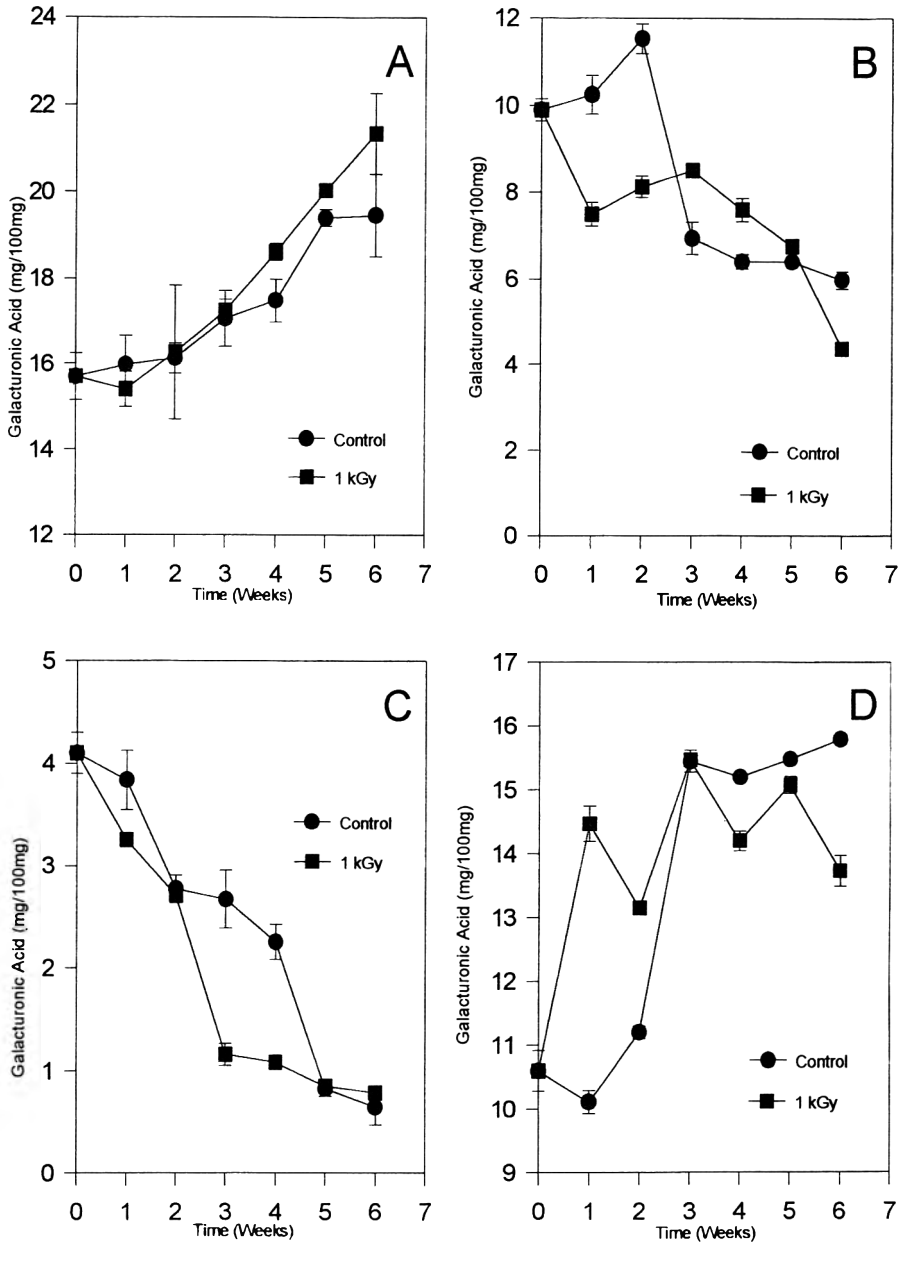

Fig. 3—Changes in concentration of (A) water soluble, (B) chelator soluble, (C) dilute alkali soluble, and (D) nonextractable pectic substances in cell walls of *pico de gallo* as affected by gamma processing (1 kGy) and storage at 2°C. Bars represent standard error of the mean.

the container than the product or microorganisms were consuming through respiration. Ethanol levels in containers did not accumulate during storage (data not shown) consistent with high oxygen levels and aerobic respiration. Ethanol concentration was not affected by gamma processing, with levels < 100 ppm throughout storage.

Solubility characteristics of pectic substances were affected by gamma processing and storage (Fig. 3). Levels of WSP increased during storage in both irradiated and non-radiated samples. Elevated levels of WSP have been typically associated with tomato softening (Huber, 1983; Thomas, 1988). Ripe red tomatoes were used for processing *pico de gallo* so the product was relatively soft and contained high levels of WSP at day 0. Elevated levels of WSP during storage were probably due to pectin degradation by polygalacturonase (Huber, 1983). Levels of CSP increased up to 2 wk storage in non-irradiated samples, and then declined sharply during the remainder of storage. In contrast, CSP levels in irradiated samples declined during the first week of storage, remained relatively constant from one to three, and then declined during the remainder of storage. The initial decline in CSP in irradiated samples may be due to cleavage of ionic bonds by free radical molecules. Reduced levels of CSP late during storage in both non-irradiated and irradiated samples may be attributed to chelation of calcium ions by citric acid. Citric acid is a major organic acid in tomatoes (Stevens et al., 1977), and has been shown to promote softening by sequestering calcium ions from cell walls (Doesberg, 1957; Buescher and Hobson, 1982). Levels of OHSP declined in both non-irradiated and irradiated samples. Dilute alkali soluble pectins are probably high molecular weight, methylated pectins. A large reduction in OHSP occurred in irradiated samples from 2–3 wk storage. This change was accompanied by a reduction in pectin DE and an increase in NXP in irradiated samples from 2–3 wk storage (Fig. 4). Nonextractable pectic substances are large molecular weight, demethylated pectic substances highly resistant to acid and enzymatic hydrolysis (Howard and Buescher, 1989). These results indicated that pectin DE was reduced in response to irradiation resulting in conversion of OHSP to NXP. Pectinmethylesterase (PME) may have been stimulated by radiation leading to reduced pectin methylation. PME activity increased in peaches, pears, cherries and citrus fruit after exposure to gamma irradiation (Somogyi and Romani, 1964; Rouse and Dennison, 1968). A large increase in NXP occurred in irradiated samples during the first week. This change was consistent with reduced levels of CSP and OHSP during this time. NXP levels in nonradiated samples increased readily

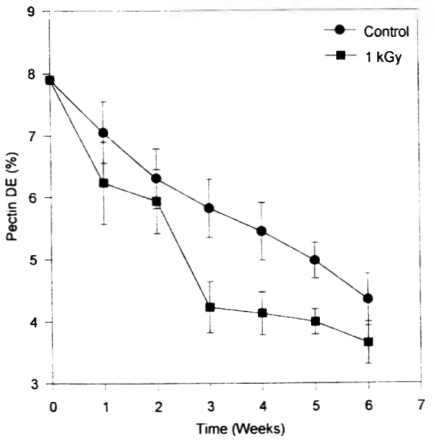

Fig. 4—Changes in pectin degree of esterification (DE) in cell walls of *pico de gallo* as affected by gamma processing (1 kGy) and storage at 2°C. Bars represent standard error of the mean.

from week one to week three and then remained relatively constant during the rest of the study. The large increase in NXP from week two to three was accompanied by a large decline in CSP. Chelation of calcium ions by natural sequesterants such as citric acid may be responsible for changes in pectin solubility.

Pectin DE values, which represent the amount of pectin molecules esterified with methanol, were low at the beginning and declined about 50% during storage. Fresh tomatoes typically have DE values from 40% at the mature green stage to 25% at ripe red stage (Huber and Lee, 1985). The low initial values for ripe tomatoes (7.9%) and changes during storage may have been due to stimulation of PME by minimal processing.

CONCLUSIONS

GAMMA PROCESSING at 1 kGy reduced populations of aerobic mesophilic and lactic microflora. Sensory attributes and headspace gases were not affected by gamma processing. Ascorbic acid content declined 50% immediately after gamma processing, but differences were slight between irradiated and non-irradiated samples at the end of storage. Pectin solubility was affected by gamma processing. Irradiation caused conversion of CSP to OHSP and NXP and a reduction in pectin DE. Gamma processing appears to be a promising treatment for extending the shelf life of refrigerated *pico de gallo*.

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Chemical, Physical and Sensory Attributes of Formed and Frozen, Baked Sweetpotato

J.L. COLLINS, J.-Y. LIAO and M.P. PENFIELD

ABSTRACT

Jumbo-size, baked sweetpotato roots of Southern Delight and Carolina Bunch (CB) cultivars were prepared as a formed, frozen product. Cultivar differences affected composition slightly. A baking regime of 204°C for 70–80 min resulted in β-carotene of 48.5 mg/100g, 2.3% greater than that at 190°C for 75–90 min. Between cultivars Hunter color values were different ($p < 0.05$). Color was an intense orange. Minor color changes occurred during 6 mo storage at −17°C, but no trend developed. CB produced the more acceptable product with greater purchase potential. Baking and storage exhibited minimum sensory effects.

Key Words: sweetpotato, β-carotene, composition, color, acceptability

INTRODUCTION

SWEETPOTATO is an important crop in the southern United States and other areas of the world (Bouwkamp, 1985; USDA, 1984b; Woolfe, 1992). It is a nutritious source of β-carotene (pro-vitamin A), ascorbic acid (vitamin C), dietary fiber, complex carbohydrates and minerals (Picha, 1986; USDA, 1984a). Its low consumption may be increased with development of ready-to-serve products.

Considerable research has been devoted to development of sweetpotato products. Hoover (1963, 1967) and Deobald and McLemare (1962) initiated the development of precooked, dehydrated flakes. Kimbrough and Kimbrough (1961) developed frozen patties. Walter and Hoover (1986) reported on an acceptable French-fried product and Bozorgmehr (1987) investigated some quality attributes of chips. Boggess et al. (1967) developed a canned sweetpotato "log" while Santos and Ammerman (1980) developed a frozen "log." Collins and co-workers investigated the use of sweetpotato in production of doughnuts (Collins and Abdul-Aziz, 1982), edible sheets (leathers, Collins and Washam Hutsell, 1987), flavored and frozen products (Collins et al., 1976), yogurt (Collins et al., 1991a,b), flour (Collins and Gurkin, 1990), pasta (Pangloli, 1993) and others.

Baking is the most widely used traditional method of cooking sweetpotato, mostly in homes and restaurants (USDA, 1984a; Woolfe, 1992). A review of baking quality of SP was published by the S-101 Technical Committee (1980) a group of sweetpotato researchers. Baking requires continuous availability of suitable raw roots, time and effort. A prepared, baked product preserved by freezing and conditioned by heating before eating should contribute to increased consumption.

Our objectives were to develop and analyze a formed and frozen, baked sweetpotato product for selected quality characteristics, acceptability and potential for purchasing as affected by cultivar, baking regime and frozen storage.

MATERIALS & METHODS

Source and preparation

Southern Delight and Carolina Bunch cultivars of sweetpotato (*Ipomoea batatas* Lam.) were used for preparation of samples. Personnel of the Plant & Soil Science Dept. produced the sweetpotatoes on the University Plant Sciences Farm, Knoxville, using standard cultural practices.

Freshly harvested jumbo-size roots were cured (held at 30°C, 80–90% relative humidity) for 10 days, stored 4 months at 14°C and prepared into samples. Washed roots were baked at 190°C for 75–90 min or 204°C for 70–80 min. Baking time varied with weights of the roots, larger roots requiring longer times. Baked roots were cooled in air to permit handling, peeled by hand and cut into irregular-shaped pieces (≈2.5 × 2.0 × 1.5 cm). The pieces were stuffed into cellulose casing (42.6-mm diameter when filled) supplied by Viskase Co. (Chicago, IL) with a Frey Electro-Hydraulic Stuffer (Koch Supplies Inc., Kansas City, MO). After filling with sweetpotato pieces, the casing was tied into 20-cm sections which were wrapped inside a general purpose, kitchen-type plastic film, frozen at −34°C in an air blast freezer, transferred to −17°C and held 0, 2 or 6 months before testing.

Chemical measurements

Proximate composition of samples was determined as follows: total dietary fiber on 1g by the enzymatic-gravimetic method of Prosky et al. (1988), moisture on 2g by vacuum oven, crude protein on 1g by Kjeldahl (N × 6.25), crude fat on 2g by petroleum ether extraction in a Goldfish apparatus and ash on 2g by muffle furnace (DWB) (AOAC, 1990). Nondietary fiber carbohydrate concentration was determined by difference, subtracting percentage of the other solids from 100%. Three replicates were analyzed with one measurement each.

β-carotene was determined on 1g sweetpotato for each of three replicates by a modification of Cort's HPLC method as presented by Holden (1985). Standard solutions consisted of 0.003 mg/mL all-trans-β-carotene (Sigma Chemical Co., St. Louis, MO) dissolved in 5, 10 and 15 mL hexane. A standard curve, prepared from duplicate injections of standard solutions, was used to determine β-carotene concentration of samples. β-carotene concentrations were converted to retinol equivalents (RE) of vitamin A by the following equation: 6 μg β-carotene = 1 RE vitamin A (NRC, 1989) and expressed as RE/100 g.

Color

A Hunter Colorimeter, model D25D2M, (Hunter Laboratories, Reston, VA) was used to measure color: L − lightness, a − redness, and b − yellowness. The colorimeter was standardized with an orange tile: L = 58.9, a = 27.9, b = 31.8 (Lanier and Sistrunk, 1979). Thawed sweetpotato product was blended in an Osterizer blender (Oster Corp., Milwaukee, WI) and filled into a cuvette with an optical glass bottom for measurement. Three replicates were measured.

Sensory evaluation

Sweetpotato samples removed from −17°C storage at 2 and 6 months were heated and evaluated by a sensory panel. Pieces (18-mm long) were cut from the frozen tubular units (42.6-mm diameter) and heated in a microwave oven (750 watts), model KM540G, (White-Westinghouse Appliance Co., Pittsburg, PA) for 4 min at full power before serving. An untrained panel consisting of 30 students and staff of agriculture-related departments evaluated the samples in a laboratory designed for sensory testing of foods (IFT Sensory Evaluation Div., 1981). Cool white fluorescent light illuminated the samples. The samples were evaluated for acceptability (color, flavor, texture and overall) on three separate days on an 8-point hedonic scale of 1 = dislike extremely and 8 = like extremely. The samples were evaluated also on a 6-point FACT scale (Schutz, 1965) for potential for purchasing the product. Panelists received one sample at a time in random order; a sample consisted of ¼ of a heated 19-mm × 42.6-mm diameter piece. Panelists were instructed to rinse their mouths with spring water between samples.

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Table 1—Proximate composition (%) of formed and frozen, baked sweetpotato product from two cultivars

Cultivar	Moisture	Protein[a]	Fat[a]	Ash[a]	TDF[a,b]	NFE[a,b]
Southern Delight	72.6c	7.9d	0.9d	5.2d	10.9c	75.0c
Carolina Bunch	71.7d	8.1c	1.1c	5.3c	10.9c	74.6d

N = 18 (baking × storage × replication; 2 × 3 × 3).
[a] Dry weight basis.
[b] Total dietary fiber; nitrogen-free extract.
[c,d] Means within a column followed by different letters are different at $p<0.05$.

Table 2—β-carotene of formed and frozen, baked sweetpotato product from two cultivars

Cultivar	β-carotene (mg/100g)	Retinol equivalents[a] (RE/100g)
Southern Delight	47.4c	7,901
Carolina Bunch	48.5b	8,076

N = 18 (baking × storage × replication, 2 × 3 × 3).
[a] mg β-carotene × 1000 ÷ 6 = retinol equivalents.
[b,c] Means followed by different letters are different at $p<0.05$.

Table 3—β-carotene of formed and frozen, baked sweetpotato product as affected by baking × storage interaction

Baking (°C/min)	Storage (months)	β-carotene (mg/100g)	Retinol equivalents[a] (RE/100g)
190/75–90	0	46.9c	7,814
	2	47.5bc	7,965
	6	47.4c	7,904
204/70–80	0	49.3b	8,211
	2	48.0bc	8,001
	6	48.0bc	8,035

N = 18 (baking × storage × replication; 2 × 3 × 3).
[a] mg β-carotene × 1,000 ÷ 6 = retinol equivalents.
[b,c] Means followed by different letters are different at $p<0.05$.

Table 4—Hunter color of formed and frozen, baked sweetpotato product as affected by cultivar or storage period

Factor	L	a	b	a/b
Cultivar				
Southern Delight	43.6b	24.3b	26.1b	0.931
Carolina Bunch	44.2a	24.6a	26.3a	0.935
Storage (months)				
0	43.9a	24.4b	26.3a	0.928
2	43.9a	24.6a	26.2b	0.939
6	43.9a	24.4b	26.1c	0.935

N = 18 for cultivar (baking × storage × replication; 2 × 3 × 3); 12 for storage (cultivar × baking × replication; 2 × 2 × 3).
[a-c] Means within a column under cultivar or storage followed by different letters are different at $p<0.05$.

All baked samples were tested on violet red bile agar (APHA, 1992) to determine if *Escherichia coli* occurred in excess of 10 colony forming units (CFU)/g. Six samples were so identified and removed from testing by sensory evaluation. These treatments included both cultivars and represented the following treatments: Carolina Bunch baked at 204/70–80 (°C/min), 6 months storage of replication 1 and 2 months storage of replication 3 and Southern Delight baked at 190/75–90 and 204/70–80 (°C/min), 2 and 6 months storage of replication 2. Thus, six of the 24 treatment-replication combinations were not evaluated.

Experimental design and analysis

For the instrumental/chemical measurements, the experiment consisted of 12 treatment combinations: two cultivars, two baking regimes, and three storage periods with three replications. Replications began as three 45-kg lots of sweetpotato, selected at the time of harvest. Data were analyzed by PROC GLM as factorials of a randomized complete block (Ott, 1988; SAS Institute Inc., 1985).

For sensory evaluations, the experiment consisted of eight treatment combinations with three replications: similar to the instrumental/chemical tests but with two storage periods, 2 and 6 months. Data were analyzed by GLM as factorials of a randomized incomplete block.

Significant differences among means were determined with the "pdiff" option of PROC GLM (SAS Institute Inc., 1985). Where GLM did not estimate the means, a one-way analysis of variance of the data was used, and where appropriate, means were separated by Tukey's Test (Linton et al., 1975; SAS Institute Inc., 1985).

Table 5—Acceptability and potential for purchase of formed and frozen, baked sweetpotato product from two cultivars, two baking regimes and frozen storage periods as evaluated by 30 panelists

Factor	Acceptability[a] Color	Flavor	Texture	Overall	Purchase[b]
Cultivar					
Southern Delight	5.2d	5.3d	5.1d	4.9d	3.1d
Carolina Bunch	6.6c	5.8c	6.2c	6.0c	3.8c
Baking (°C/min)					
190/75–90	5.7d	ns	ns	ns	ns
204/70–80	6.0c				
Storage (months)					
2	ns	5.4d	ns	ns	3.3d
6		5.7c			3.6c

[a] 5 = like slightly; 6 = like moderately; 7 = like very much.
[b] 3 = would buy occasionally; 4 = would buy now and then.
[c,d] Means within a factor and column followed a different letters are different at $p<0.05$.

RESULTS & DISCUSSION

CONCENTRATION OF PROXIMATE COMPOSITION, except total dietary fiber, was affected ($p < 0.05$) by cultivar, but not by baking regime or frozen storage time. Samples of Carolina Bunch had higher concentrations of crude protein, crude fat and ash and a lower concentration of moisture and nitrogen-free extract (Table 1). Total dietary fiber did not differ between cultivars. Overall, the baked sweetpotato product contained relatively low concentrations of protein, fat and ash, but important concentrations of dietary fiber. Its nutritional value was similar to that reported for conventionally baked sweetpotato (USDA, 1984a).

Cultivar, baking regime and baking regime × storage interaction affected ($p < 0.05$) β-carotene concentration. That for the product prepared with Carolina Bunch was 48.5 mg/100g (DWB), 2.3% higher than that from Southern Delight (Table 2). When converted to RE, the average of both cultivars was 7,988 RE/100g (DWB) which agreed closely with 8,036 RE/100g of conventionally baked sweetpotato, (USDA, 1984a). Differences in β-carotene concentration between and within cultivars occur from small to large proportions (Bouwkamp, 1985).

Over the 6-months storage, there was a tendency for an increase in β-carotene by baking at 190°C for 75–90 min, but a decrease by baking at 204°C for 70–80 min (Table 3). On average, β-carotene was greater when the sweetpotatoes were baked at 204°C for 70–80 min. The greater β-carotene concentration was related to shorter baking times rather than to lower temperatures.

Cultivar affected ($p < 0.05$) all Hunter color values, and storage time affected Hunter a and b. Baking regime did not affect color. All Hunter values for Carolina Bunch were higher ($p < 0.05$) than values for Southern Delight; however, the magnitude of differences was small (Table 4). The range of Hunter a and b values that occurred during storage, although significant, was of little practical importance. The a/b ratio was 0.931 for Southern Delight and 0.935 for Carolina Bunch, indicating that the color was a strong orange.

The differences among Hunter a or b values in the frozen product over 6-months were small (Table 4). The average a value (redness) was higher by 0.2 units after 2 months storage while the average b value was highest at 0 months storage and decreased by 0.2 units during 6 months storage.

The correlation between Hunter b and β-carotene in the frozen product during storage was $r = -0.8205$. Over the 6-mo period, Hunter b decreased 0.2 units while β-carotene increased 3.6%. We hypothesized, that a reduction in yellowness would be directly related to decreased β-carotene. Thus the decrease in yellowness was expected, but an increase in β-carotene in the baked, frozen product was not readily explained.

Differences between cultivars affected ($p < 0.05$) all acceptability characteristics and the likelihood to purchase the product. Baking regime affected color only while storage time affected flavor and purchase potential. The panelist factor was significant ($p < 0.05$) for all attributes and potential for purchasing, indicating that differences existed among panelists in their evaluations. Samples of Carolina Bunch were more acceptable and more likely to be purchased than Southern Delight (Table 5). Overall, scores for samples of both cultivars ranged between 4.9 and 6.6, where 5 = liked slightly and 7 = liked very much. Carolina Bunch would be purchased "now and then," while Southern Delight would be purchased "occasionally." Samples baked at 204/70–80 (°C/min) were more acceptable for color only, in agreement with a higher concentration (by significance or tendency) of β-carotene (Table 3) in samples baked at higher temperatures (baking regime was significant). Samples stored 6 months were more acceptable for flavor and more likely to be purchased. These results indicate that the baked products when held frozen up to 6 months retained sensory quality attributes.

CONCLUSIONS

THE ATTRIBUTES of formed and frozen, baked sweetpotato products met with sensory panel acceptance. The products retained original attributes for 6 months storage. Differences between cultivars affected all measurements, whereas baking regime and frozen storage had lesser effects. The textural integrity of samples apparently was similar to that of the baked root. Size, shape and quantity of individual units of finished products were under the control of the producer. Jumbo-size roots, the least costly of all sizes, were desirable for use as this size is produced in adequate quantities and finds limited utility.

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Enzymatic Maceration of Vegetables with Protopectinases

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ABSTRACT

Protopectinases (PPases) are a heterogeneous group of enzymes that solubilize pectin from the insoluble protopectin in plant tissues by restricted depolymerization. Various PPases have been reported with different pectin solubilizing activities depending on the substrate. We studied the macerating properties of some PPases on potato, carrot, garland chrysanthemum, garlic, ginger, spinach, and red pepper. Crude culture filtrate from *Trichosporon penicillatum* SNO-3, containing PPase-SE, was most effective for maceration of potatoes, and the PPase-B from *Bacillus subtilis* IFO 12113 was most effective for carrots. Enzyme concentration, pH, reaction time, and rate of shaking affected the yield of single cells.

Key Words: maceration; protopectinase; polygalacturonase; potato; carrot

INTRODUCTION

ENZYMATIC PREPARATIONS that disintegrate plant tissues are used for processing fruits and vegetables. They are classified into two groups according to extent of disintegration (Rombouts and Pilnik, 1978). One group contains those mixtures that degrade polygalacturonic acid chains and therefore can totally disintegrate plant tissues. They are used mainly in production of foodstuffs with high proportions of soluble solids like tomato paste or puree, and also to improve yields in fruit juice production. Usually, a combination of cellulolytic and pectolytic enzymes is needed to achieve almost complete liquefaction.

The other group includes the "macerating" enzymes, which can produce a suspension of loose single cells and are used to prepare fruit nectar bases, vegetable purees, and baby and geriatric foods. For such purposes, only the intercellular cementing material that holds together cells and some portion of primary plant cell walls should be removed without damage to adjacent secondary cell walls (to help avoid cell lysis). For this reason, cellulases in the enzyme mixture are undesirable.

The intercellular cementing material of plant tissues is called the middle lamella (Fry, 1988). Both the middle lamella and primary cell walls have complex chemical compositions with protopectin a main component. Protopectin is an insoluble pectic substance found in plant tissues; it becomes soluble after restricted degradation. In a proposed model of the chemical structure of protopectin, neutral sugar side chains are arranged in blocks (hairy regions) and connect unsubstituted regions, which are composed almost exclusively of partially methoxylated galacturonic acid residues (smooth regions), with cellulose fibers (Gullion et al., 1989; Renard et al., 1990). Each molecular component of protopectin is potentially susceptible to attack by a specific enzyme. The term protopectinases (PPases) originally referred to enzymes that hydrolyze or dissolve protopectin, liberating water-soluble pectin with the resultant separation of plant cells from each other (Brinton et al., 1927). Thus, PPases

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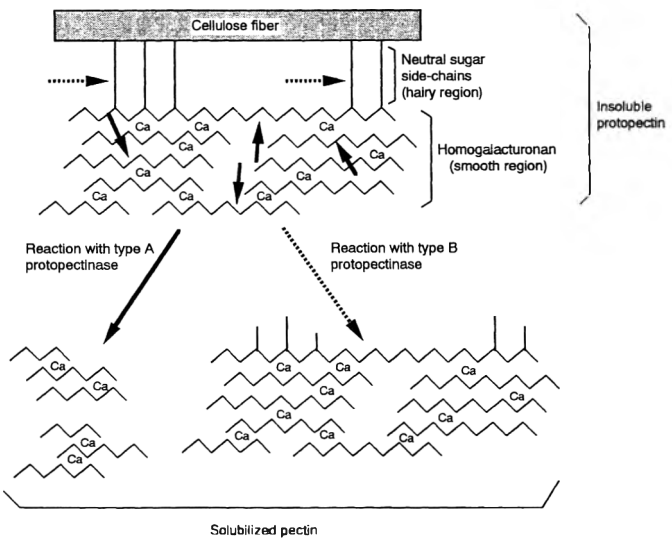

Fig. 1—Postulated structure of protopectin and reaction sites of type A and type B protopectinases.

are a heterogeneous group of enzymes with different catalytic activities. Among them, pectolytic enzymes, which degrade the smooth regions, and glycan hydrolases, which degrade the hairy regions, are the most important.

Sakai and Okushima (1980) first reported the enzymatic solubilization of pectin from citrus peel protopectin. Several PPases have since been isolated and characterized (Sakai and Okushima, 1982; Sakai et al., 1982, 1984, 1989; Sakai and Yoshitake, 1984; Sakai and Takaoka, 1985; Sakai and Sakamoto, 1990; Sakamoto et al., 1994a; Yoshitake et al., 1994), and they have been classified into two types depending on reaction mechanisms (Sakai, 1992; Sakai et al., 1993). Type A and type B PPases react with smooth and hairy regions of protopectin (Fig. 1).

PPases have one common characteristic. They are much more active against insoluble forms of specific substrates, which is desirable for enzymes used in maceration. Type A PPases with the activities of polygalacturonase, pectate lyase, or pectin lyase release soluble pectin by depolymerizing the smooth regions in protopectin. Because of that characteristic such enzymes liberate soluble pectin from protopectin without degrading solubilized pectin at the same time. Type B PPases do not degrade pectin molecules, and may react on polysaccharide chains that connect the pectin molecule to cell wall polysaccharides.

Our objective was to study the maceration of various typical plant materials using different crude culture filtrates containing PPase activity of either bacterial or fungal origin. In some cases, effects of variables such as pH, enzyme concentration, reaction time, and shaking on maceration and final yield of single cells were included.

MATERIALS & METHODS

Chemicals

All chemicals were obtained from Wako Pure Chemicals Industries (Osaka, Japan) unless otherwise stated, and were of certified reagent grade.

Table 1—Microorganisms producing PPase used for maceration tests

Microorganism	Name of PPase[a]	Type of activity	Reference
Trichosporon penicillatum SNO-3	PPase-SE	Endo-PGase	Sakai and Okushima (1982)
Kluyveromyces fragilis IFO 0288	PPase-F	Endo-PGase	Sakai et al. (1984)
Aspergillus kawachi IFO 4308	PPase-AK	Unknown	Unpublished
Aspergillus awamori IFO 4033	PPase-AW	Unknown	Unpublished
Bacillus subtilis IFO 12113	PPase-B	Endo-PGL	Sakai et al. (1989)
Bacillus subtilis IFO 3134	PPase-C	Arabinase	Sakai and Sakamoto (1990)
	PPase-N	Endo-PGL	Sakamoto et al. (1994a)
	PPase-R	Endo-PMGL	Sakamoto et al. (1994a)

[a] PPase: protopectinase. PGase: polygalacturonase. PGL: polygalacturonate lyase. PMGL: polymethylgalacturonate lyase.

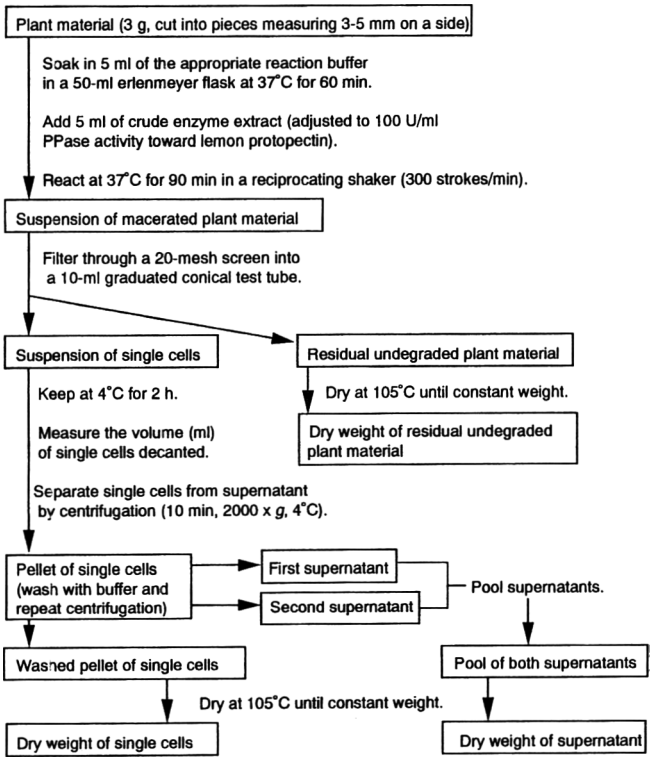

Fig. 2—Methodology used in maceration tests of different plant materials. PPase, protopectinase.

Enzyme sources

Crude filtrates containing PPase activity from different liquid cultures of bacterial or fungal strains were used for maceration tests. The microorganisms used were previously screened for selection of those that produced enzymatic pools with highest PPase activities (Table 1). These microorganisms have long been used in the Japanese food industry, and are generally considered safe. Further information about screening and culture conditions are in the references, and for *Aspergillus* strains, details will be published elsewhere. *Bacillus subtilis* IFO 3134 culture filtrates used in the experiment contained three PPases, -C, -N, and -R, with different reaction mechanisms. The activities of PPase-N and -R were much lower than that of PPase-C, so the total enzymatic pool is labeled PPase-C for simplicity.

Assay of PPase activity

PPase activity in the enzyme preparation was assayed by measurement of pectic substances liberated from lemon protopectin by the carbazole-H_2SO_4 method (McComb and McCready, 1952) with D-galacturonic acid monohydrate (Sigma Chemical Co., St. Louis) as standard. The reaction was at 37°C for 1 hr in a total volume of 1 mL containing 10 mg of protopectin and the enzyme diluted appropriately in buffer. The buffer pH was selected to obtain optimum PPase activity on lemon protopectin. A 20 mM acetate buffer pH 5.0 was used for PPase-SE and -F. A 20 mM potassium phosphate buffer pH 6.0 was used for PPase-C but for PPase-AK and -AW the pH was 6.5 (unpublished data), and for PPase-B it was 8.5. Lemon protopectin was prepared as described (Sakamoto et al., 1994a). One unit of PPase activity was defined as that amount that liberates soluble pectic substances corresponding to 1 mmol of D-galacturonic acid in 1 h under these reaction conditions.

Table 2—Volume of single cells produced by treatment of various plant materials with different PPases[a]

PPase	Reaction pH	Volume of single cells formed (mL) Po	Ca	Gd	Ga	Gi	Sp	Rp
-SE	5.0	3.8	2.9	2.5	0.8	0.3	0.1	3.1
-F	5.0	0.6	1.3	0.6	0.9	0.1	0.1	1.9
-B	8.5	3.3	2.0	2.1	1.0	0.1	0.5	2.9
-C	6.0	1.3	0.5	0.5	0.2	0.1	0.5	ND
-AK	6.5	2.3	0.2	0.1	1.0	0.1	0.1	2.3
-AW	6.5	1.8	0.3	0.1	0.1	0.1	0.1	1.9

[a] PPase: protopectinase. Po: potato. Ca: carrot. Gd garland chrysanthemum. Ga: garlic. Gi: ginger. Sp: spinach. Rp: red pepper. ND: not determined. Experimental procedures are described in the text and in Fig. 2.

Table 3—Results of maceration tests with proportions of the different fractions obtained (expressed as a percentage of total dry solids) from plant materials treated with PPase-SE[a]

Plant material	Insoluble residue (%)	Soluble substances (%)	Single cells (%)
Potato	27	13	60
Garlic	4	60	36
Ginger	72	23	5
Red pepper	35	48	17

[a] Experimental procedures are described in the text and in Fig. 2.

Table 4—Results of maceration tests with proportions of the different fractions obtained (expressed as a percentage of total dry solids) from plant materials treated with PPase-B[a]

Plant material	Insoluble residue (%)	Soluble substances (%)	Single cells (%)
Potato	41	19	40
Garlic	6	60	34
Ginger	61	36	3
Red pepper	42	40	18

[a] Experimental procedures are described in the text and in Fig. 2.

Assay of maceration activity

Different fresh plant materials purchased in a local market were used for tests of maceration with crude culture filtrates which had PPase activity. Potato (*Solanum tuberosum* L. cv. Danshaku), carrot (*Daucus carota* L. var. *sativa* DC.), garland chrysanthemum (*Chrysanthemum coronarium* L.), garlic (*Allium sativum* L. var. *japonicum*), ginger (*Zingiber officinale* Rosc. var. *macrorhiza*), spinach (*Spinacia oleracea* L.), and red pepper (*Capsicum annuum* Irish var. *fasciculatum*) were chosen because of the potential interest of their macerates to the food industry. Potato tubers, carrot roots, garlic bulbs, and ginger rhizomes were peeled and cut into pieces measuring 3–5 mm on each side. Seeds were removed from red pepper, and the garland chrysanthemum leaves and spinach leaves were used whole. These samples were also cut into pieces measuring 3–5 mm on a side.

Maceration tests consisted of a series of suspensions, filtrations and centrifugations (Fig. 2). Unless otherwise stated, 50 PPase U/mL of reaction mixture was used and the buffer pH was chosen to obtain optimum PPase activity. As a control, blanks were prepared with heat-denatured enzymes. Different buffers were used in assays of the effects of pH on maceration activity: 20 mM acetate buffer for pHs 4.0

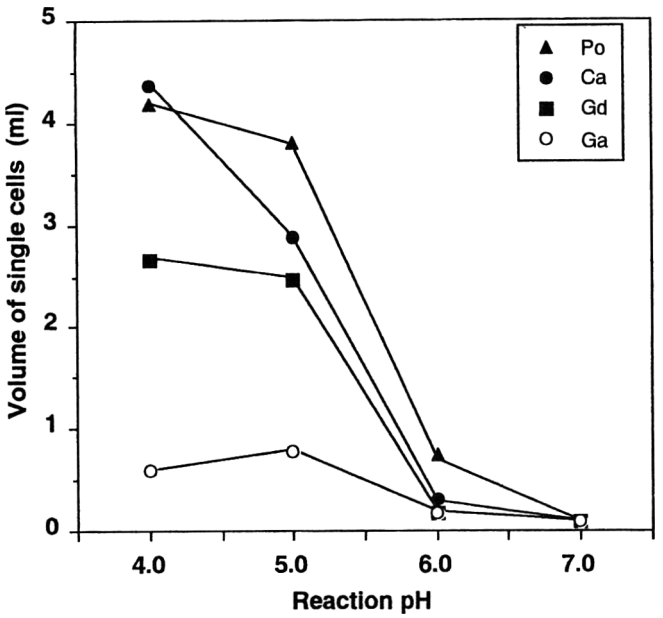

Fig. 3—Effects of reaction pH on the maceration of different plant materials with crude culture filtrates of *Trichosporon penicillatum* SNO-3, containing PPase-SE activity. The reaction mixtures contained 50 PPase-SE U/mL. Experimental procedures are described in the text and in Fig. 2. Po: potato, Ca: carrot, Gd: garland chrysanthemum, Ga: garlic.

and 5.0, and 20 mM potassium phosphate buffer for pHs 6.0 and 7.0. The volume of single cells produced (mL of single cells decanted) was measured. At times, when PPase-SE and -B were used for maceration tests, the residual undegraded plant material, the supernatant of the suspension of single cells, and the pellet of single cells were dried at 105°C to constant weight and the dry weight recorded. Five reaction mixtures were tested and the mean of the values was recorded.

RESULTS & DISCUSSION

THE MACERATION ACTIVITY of different crude extracts containing a PPase belonging either to type A (PPase-SE, -F, -B, -N, or -R) or to type B (PPase-C) were compared on the plant materials (Table 2). All maceration blanks yielded negligible values. Maceration activity of different PPases varied greatly. The values could be analyzed in two ways. We could consider the enzyme specificity (maceration of same plant material by different enzymes) or the substrate specificity (the ability to produce single cells from different substrates by the same enzyme). The yield of maceration of red pepper was high with all enzymes tested, so the middle lamella of that plant could be degraded readily by different enzymatic mechanisms. Potato was easily macerated except by PPase-F, but carrot and garland chrysanthemum yielded large amounts of single cells only with PPase-SE and -B. Garlic was macerated with difficulty and only with PPase-B, -AK, -F, and -SE. Ginger and spinach were highly resistant to maceration with all PPases. PPase-SE gave the largest amount of single cells from potato, with intermediate yields of red pepper, carrot, and garland chrysanthemum, and low yields of garlic, ginger, and spinach. PPase-B gave a high yield from potato, intermediate yields from red pepper, garland chrysanthemum, and carrot, and low yields of garlic, spinach, and ginger. Other PPases had lower maceration activities than these two enzymes, so PPase-SE and -B were used for further maceration tests.

Amounts of dry solids were compared in residual undegraded plant material, pooled supernatants, and pellets of single cells formed after maceration of some vegetables by PPase-SE (Table 3) and -B (Table 4). This would be important because the main purpose of enzymatic maceration is to maximize conversion of plant tissues into single cells. Consequently, amounts of both

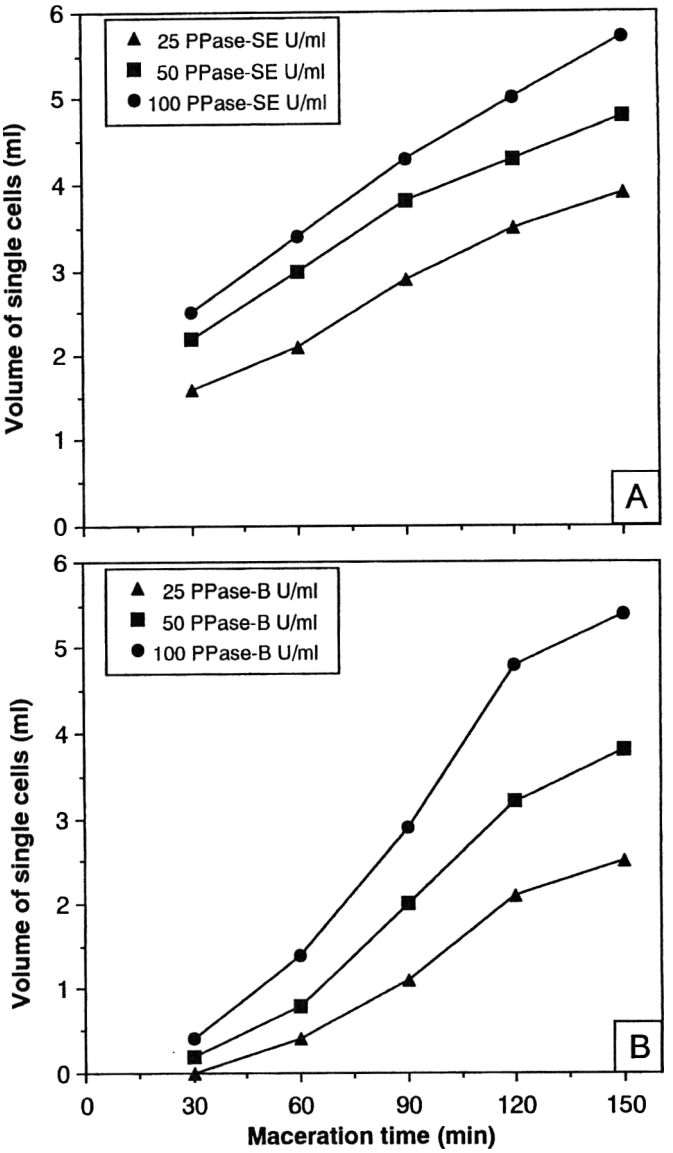

Fig. 4—Changes with time during the maceration of potato (A) and carrot (B) with crude culture filtrates of *Trichosporon penicillatum* SNO-3 and *Bacillus subtilis* IFO 12113 with different concentrations of PPase-SE and -B, respectively. Experimental procedures are described in the text and in Fig. 2.

the plant material that resisted enzymatic reation and the solubilized solids liberated as a consequence of cell damage should be minimized to optimize yield. The proportion of solids in the single cells from potato samples was high, particularly with PPase-SE, and the proportion of insoluble (undegraded) and soluble solids were low. Therefore, the efficiency of this enzyme in macerating potato, expressed as percentage of solids in original material that was found in single cells after digestion, was the highest found. The opposite results were observed in ginger samples, in which most of the initial material remained as an insoluble residue. An interesting result was observed in the maceration of garlic. Although the proportion of solids in the insoluble residue was very low, the yield of single cells produced was not high because many were lysed, as was evident by the high proportion of soluble substances released during maceration.

Effects of buffer pH during maceration were compared on different plant materials with PPase-SE (Fig. 3). Maceration activity was maximum at pHs 4.0 to 5.0. As reported (Sakai et al., 1982), PPase-SE is an endopolygalacturonase with optimum activity at pH 5.5 when assayed on soluble polygalacturonic acid, and only 20% of this activity was detected at pH 4.0. Moreover,

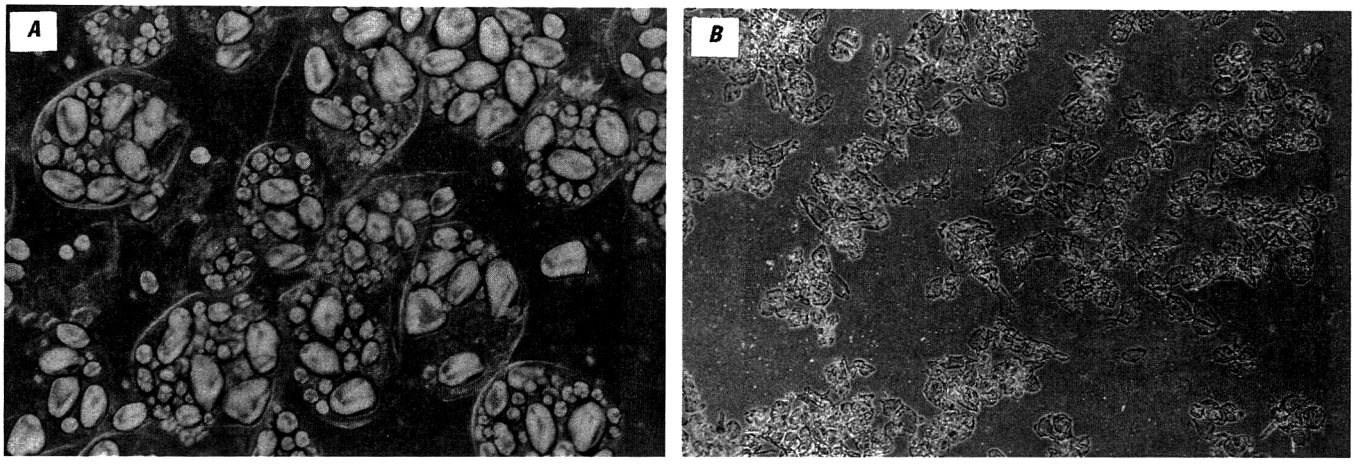

Fig. 5—Photomicrographs of the macerates obtained from potato (A, 200 X) and carrot (B, 80 X) with crude culture filtrates of *Trichosporon penicillatum* SNO-3 and *Bacillus subtilis* IFO 12113, containing PPase-SE and -B, respectively. The reaction mixtures contained 50 PPase U/mL. Experimental procedures are described in the text and in Fig. 2.

the optimum pH of protopectinase activity of PPase-SE is 5.0, and <10% of maximum activity was detected at pH 4.0. The reasons for these differences in such effects of pH remain unknown. Differences in physical characteristics of soluble substrates (polygalacturonic acid) and insoluble substrates (lemon protopectin or different protopectins contained in fresh plant tissues) seemed to be related to differences in the effect. In particular, adsorption onto and diffusion into the tested solid substrates by PPase-SE could be affected to different degrees by the reaction pH. Changes in net charge of chemical constituents of plant cell walls other than smooth regions in protopectin could affect the enzyme activities. None of the plant materials were blanched, so the large amount of single cells at pH 4.0 could be accounted for in part by the activity of endogenous enzymes at that pH.

Changes with time during maceration of potato with PPase-SE (Fig. 4A) and during maceration of carrot with PPase-B (Fig. 4B) were compared with different concentrations of enzyme. In the first case, many single cells were produced during the first 30 min with all 3 concentrations. Again, the presence of endogenous enzymes during soaking could contribute to the activity. Potato pectinesterase (Bartolome and Hoff, 1972) might remove methoxyl groups in the smooth regions in protopectin, increasing the number of sites for PPase-SE activity. Potato polygalacturonase could be responsible for the large numbers of single cells released early. Nevertheless, such endogenous enzymes unassisted did not macerate potato because reaction blanks showed negligible maceration. Therefore, we assume that the enzymes had a synergistic effect on the maceration process, with the addition of PPase-SE necessary for release of single cells. Later during the reaction, single-cell volumes increased almost linearly. The final yields of single cells released by PPase-SE did not level off, suggesting that longer reaction times may be necessary to achieve maximum maceration yields. The rate of release of single cells was not proportional to PPase-SE concentration. These results were in accordance with the previous report that maceration of potato was limited by the diffusion of the enzyme into the solid substrate (Biekman, 1992).

A lag phase was observed during the first 30 min in the maceration of carrot by PPase-B. Up to 60 min, the enzyme concentration and the amount of single cells produced were proportional with all 3 concentrations tested, and up to 90 min with 25 and 50 PPase-B U/mL. The maceration rate increased slightly up to 120 min. This rate did not seem limited by the diffusion of the enzyme through carrot tissue. Unlike PPase-SE, the curves tended to level off after 120 min.

Photomicrographs of macerates obtained from potato with PPase-SE (Fig. 5A) and from carrot with PPase-B (Fig. 5B) were compared. Cell walls and contents were intact in a high proportion of single cells, which had visible cytoplasmic inclu-

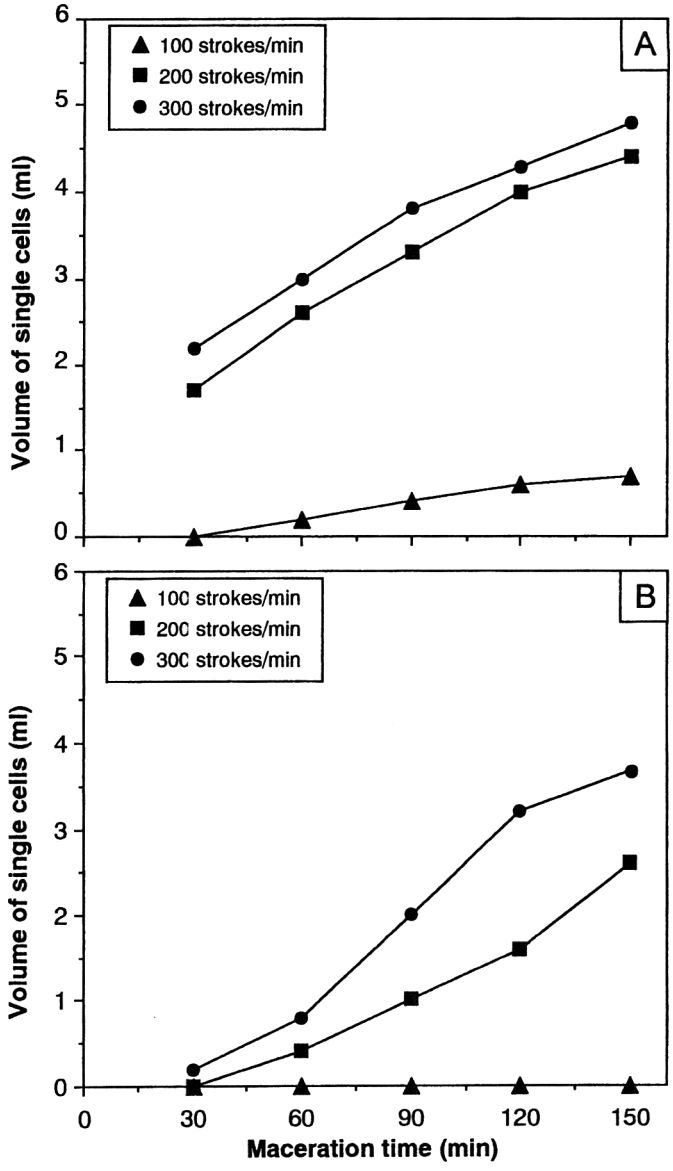

Fig. 6—Effects of shaking on the changes with time during maceration of potato (A) and carrot (B) with crude culture filtrates of *Trichosporon penicillatum* SNO-3 and *Bacillus subtilis* IFO 12113, containing PPase-SE and -B, respectively.

sions (starch and carotene particles, vacuoles, etc.). Small aggregates of cells were found as well.

Effects of shaking on kinetics of single-cell production from potato with PPase-SE (Fig. 6A) and from carrot with PPase-B (Fig. 6B) were also compared. With PPase-SE, the rate of maceration at 100 strokes/min was low, and there was little difference in results with 200 and 300 strokes/min. With PPase-B, single cells were not released at 100 strokes/min, and the reaction rate with 200 strokes/min was about 50% that at 300 strokes/min up to 120 min. Once partial depolymerization of the middle lamella had occurred, a shear force was needed to transform the plant material into a suspension of loose cells. The magnitude of the required force depended on the degree of enzymatic modification of protopectin, i.e., on the number of chemical linkages remaining that attached the plant cells to each other. The shear stress needed to release single cells from carrot was higher than that needed for potato.

The maceration of plant tissues is an example of heterogeneous catalysis in which the kinetic and thermodynamic constants of the reactions with insoluble substrates are characteristic, and different from those in homogeneous catalysis. These constants depend on enzyme-substrate interactions, which may be strongly influenced by the micro-structure of the solid substrate. Such interactions with different microenvironments in which the reactions occur are called 'locus' effects and may be either steric or electrostatic (McLaren and Packer, 1970). There may be an equilibrium partition of free PPase between the bulk phase (outside solution) and the solid phase. Some reports have discussed interactions of cellulases (Mo and Hayashida, 1988) and raw-starch-degrading amylases (Hayashida et al., 1989) with solid substrates, onto which they absorb. The enzyme mechanisms of PPases seem to be different because protopectin is a low density (not very compact) material and may be more penetrable by enzymes. Thus, the reaction rate would be limited by the diffusion of both the enzyme and the solubilized pectin (product) through the tissue. The ability to release pectin from different plant tissues depends on both the type of PPase and the plant material used as substrate (Sakamoto et al., 1994b). Consequently, the ability to release pectin from protopectin, leading to the maceration of plant tissues, depends on two main factors. Both the chemical structure of the substrate and the ability of the enzyme to reach and degrade the specific site where the reaction takes place are important in the maceration process.

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Flavonoids and Antioxidant Activity of Fresh Pepper (*Capsicum annuum*) Cultivars

Y. LEE, L.R HOWARD, and B. VILLALÓN

ABSTRACT

Flavonoid, ascorbic acid and total phenolic content and antioxidant activity of "jalapeño" (Veracruz, Mitla, Tam Mild, Jaloro, Sweet Jalapeño), "yellow wax" [(Hungarian Yellow, Long Hot Yellow, Gold Spike (hybrid)], "chile" (New Mexico-6, Green Chile), "ancho" (San Luis Ancho), and "serrano" (Hidalgo) peppers were investigated at green or yellow stages of maturity. Major pepper flavonoids were quercetin and luteolin which were present in conjugate forms. Total flavonoid content varied from none detectable to 800 mg/kg after hydrolysis. "Chile", "yellow wax" and "ancho" peppers had greater flavonoid and ascorbic acid contents and antioxidant activities than "jalapeño" peppers. Sep-Pak C_{18}™ bound phenolic compounds, including flavonoids, correlated well with antioxidant activity (r^2=0.86). Luteolin had highest antioxidant activity followed by capsaicin and quercetin on equimolar basis.

Key Words: peppers, antioxidants, flavonoids, ascorbic acid

INTRODUCTION

PEPPERS are a good source of vitamins A and C, which are important dietary antioxidants. Levels of these compounds may be affected by maturity, genotype and processing (Howard et al., 1994). Organic compounds in fruits and vegetables may be important in cancer chemoprevention (Wattenberg, 1985; Huang et al., 1994). Flavonoids are widely distributed in plants with many biochemical and pharmacological effects, including antioxidation, antiinflammation and antiallergy (Havesteen, 1983). Several reports linked flavonoid intake to reduced cancer risk (Verma et al., 1988; Yoshida et al., 1990). Flavonoids inhibited enzymes such as prostaglandin synthase, lipoxygenase and cyclooxygenase, closely related to tumorigenesis (Laughton et al., 1991; Yoshimoto et al., 1983; Smith and Yang, 1994; Bauman et al., 1980). In addition, flavonoids may also induce detoxifying enzyme systems such as glutathione S-transferase (Smith and Yang, 1994). Many kinds of flavonoids have been reported in fruits and vegetables, and types and content vary with cultivar and maturation (Hertog et al., 1992a; Sukrasno and Yeoman, 1993). From more than 30 fruits and vegetables studied, onion (284-486 mg/kg), kale (110 mg/kg), broccoli (30 mg/kg), french bean (32-45 mg/kg) and slicing bean (28-30 mg/kg) had greatest quercetin contents (Hertog et al., 1992b). Flavone content was low compared with flavonol content except fresh broad bean (25 mg myricetin/kg) and red bell pepper (13-31 mg luteolin/kg; Hertog et al., 1992). In plant cells, flavonoids occur as glycosides, with sugars bound typically at the C_3 position. Flavonoid glycoside conjugates are degraded to aglycones by human intestinal flora after ingestion (Leighton et al., 1992).

As consumption increases, peppers may contribute notable amounts of vitamins A and C, and flavonoids to the diet. Information on flavonoid composition in different pepper types and cultivars is scarce. Our objectives were to determine the levels of quercetin, luteolin and ascorbic acid in several pepper types, and to explore relationships between flavonoid content and antioxidant activity.

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MATERIALS & METHODS

Materials

Peppers (*Capsicum annuum* L.) were grown at the Texas Agricultural Experiment Station in Weslaco, TX "jalapeño" [cv Tam Veracruz, Mitla, Tam Mild, Jaloro, Sweet Jalapeño], "yellow wax" [cv Hungarian Yellow, Long Hot Yellow, Gold Spike (hybrid)], "Chile" [cv New Mexico-6, Green Chile], "ancho" [San Luis Ancho], "serrano" [cv Hidalgo] were harvested at mature green or yellow stages and shipped to Texas A&M University, College Station, TX for analysis. Quercetin was purchased from Sigma Chemical Co., (St Louis, Mo.) and luteolin was purchased from Roth Chemical Co., (Karlsruhe, Germany).

Flavonoid analysis

Flavonoid glycoside extraction and analysis was similar to that of Hertog et al. (1992b), with flavonoid hydrolysis modification. Flavonoid aglycones were analyzed after acid hydrolysis of flavonoid conjugates. A Waters model 996 photodiode array detector was used to record UV spectra of flavonoids. Chromatograms were compared with quercetin and luteolin standards.

For sample analysis, following stem and seed removal, fresh pepper samples (5g) were homogenized in 25 mL of 60% methanol, filtered through miracloth, washed with 50% methanol and adjusted to 60 mL. Extracts for quercetin assay were hydrolyzed with 1N hydrochloric acid (HCl) in 50% methanol (MeOH) at 90°C for 30 min and for luteolin analysis, extracts were hydrolized with 2N HCl in 50% MeOH at 90°C for 2 hr. Flavonoid aglycones were quantified at 370 nm using a C_{18} column (Nova-Pak, 4 mm × 15 cm), with a solvent system of MeOH/Water (45:65), pH 2.4 with phosphoric acid at 1mL/min. Recovery rates were: quercetin 96%, luteolin 75%.

Phenolic compounds

The procedure for extraction and measurement of total soluble phenolics was that of Swain and Hillis (1959). Phenolics binding to Sep-Pak™ C_{18} cartridges were isolated by evaporating ethanol extracts (80%) at 40°C, and resolubilizing in deionized water. Samples (5mL) were loaded into Sep-Pak C_{18} cartridges preconditioned with 4 mL methanol and 10 mL deionized water. After washing with 5 mL water, Sep-Pak C_{18} cartridges were eluted with 5 mL of methanol and the methanol fraction was assayed for total phenolics using chlorogenic acid as standard.

Antioxidant assay

Pepper fruit (5 g) was isolated from seed and stem tissue and homogenized in 20 mL of 80% ethanol. After centrifugation at 6000 × *g* for 10 min, 5 mL of supernatant was evaporated at 40°C. Sep-Pak™ C_{18} cartridges were used to remove polar interfering compounds such as ascorbic acid. Sep-Pak C_{18} cartridges were preconditioned with 5 mL of methanol and 10 mL of water. After sample loading (5 mL), Sep-Pak cartridges were washed with 4 mL of water and lipophilic compounds were eluted with 4 mL of methanol. Aliquots (40 µL) of the methanol fraction were assayed for antioxidant activity. Evaluation of antioxidant activity based on coupled oxidation of β-carotene and linoleic acid was conducted as described by Taga et al. (1984) with some modifications. β-carotene (2 mg) was dissolved in 20 mL of chloroform solution. A 3 mL aliquot of β-carotene chloroform solution was added to a conical flask along with 40 mg linoleic acid and 400 mg Tween 40. Chloroform was removed using a rotary evaporator at 40°C. Oxygenated distilled water (100 mL) was added to the β-carotene emulsion and mixed well. Aliquots (3 mL) of oxygenated β-carotene emulsion and 40 µL of pepper extract were placed in test tubes and mixed well. The tubes were immediately placed in a water bath and incubated at 50°C. Oxidation of β-

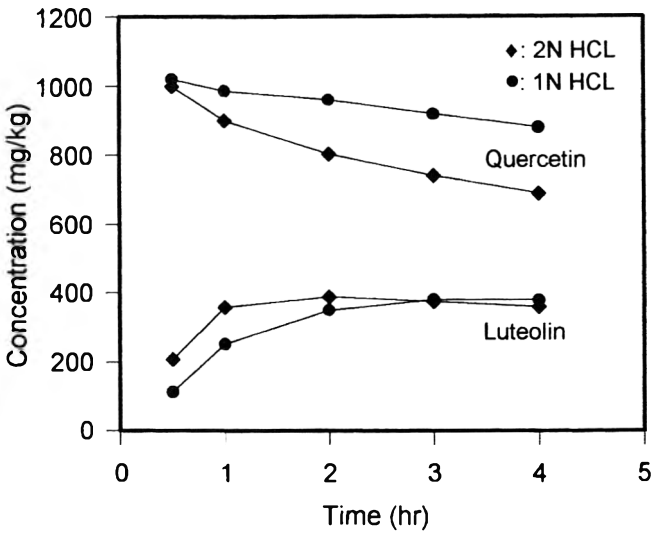

Fig. 1—Influence of acid concentration (1 and 2N HCl) and reaction time (0.5, 1, 2, 3, 4 hr) at 80°C on quercetin and luteolin yield in pepper fruit.

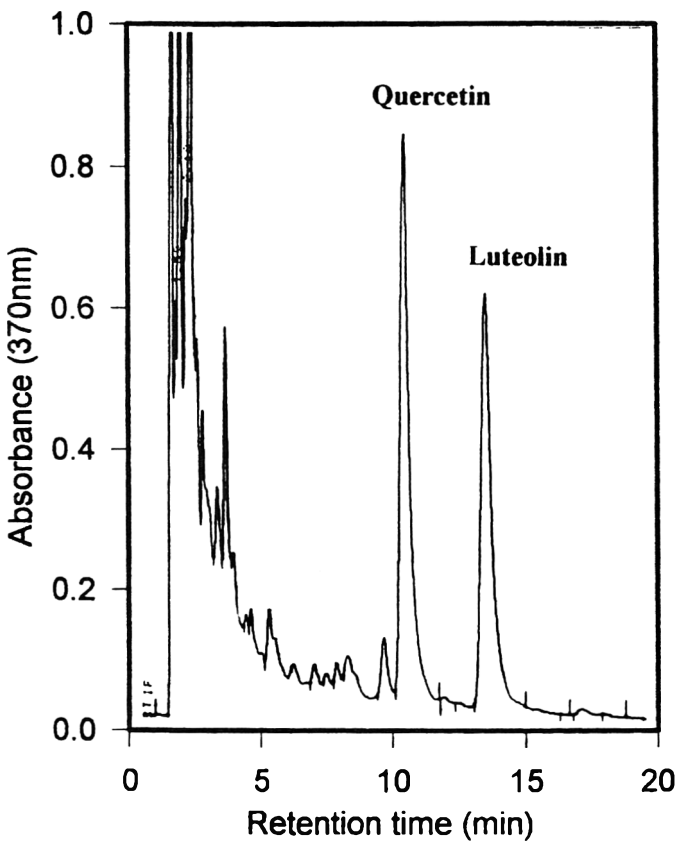

Fig. 2—Typical HPLC chromatogram of 2N HCl pepper hydrolyzate, monitored in MeOH/H_2O (45:65), pH 2.4 with phosphoric acid. Detection at 370 nm; flow rate 1 mL/min.

carotene emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm. Sample absorbance was measured 10, 20 and 30 min. after addition of oxygenated water and incubation at 50°C. A control sample consisted of 40 µL methanol, instead of pepper extract, and 3 mL of β-carotene emulsion. The degradation rate of β-carotene was calculated by first order kinetics. Antioxidant activity was expressed as % inhibition relative to the control using the equation:

$$\frac{\text{dr cont} - \text{dr samp}}{\text{drc}} \times 100$$

Table 1—Flavonoid content and heat index of fresh pepper fruit[a]

Type	Cultivar	Quercetin	Luteolin	Total flavonoids[b]	Heat index[c]
		(mg/kg)			
Jalapeno	Veracruz	nd.	nd.	nd.	7
	Mitla	39.57 f[d]	13.67 f	53.24 e	8
	Tam Mild	17.60 f	9.77 f	27.37 ef	4
	Jaloro	151.20 de	37.50 e	188.7 d	8
	Sweet Jalapeno	45.33 f	6.07 fg	51.4 e	1
Yellow Wax	Hungarian Yellow	783.83 a	67.70 b	851.53 a	5
	Long Hot Yellow	446.67 b	103.50 a	550.17 b	7
	Gold Spike (Hybrid)	288.33 c	36.83 e	325.16 c	5
Chile	New Mexico 6	125.67 e	50.57 cd	176.24 d	5
	Green Chile	210.23 d	51.53 c	261.76 d	5
Ancho	San Luis Ancho	276.00 c	33.63 e	309.63 c	5
Serrano	Hidalgo	159.80 de	41.4 de	201.2 d	6

[a] Maturity is green mature stage except yellow type peppers.
[b] Total flavonoids = quercetin + luteolin.
[c] Heat index was obtained from Villalon (1994).
[d] Means in each column with similar letters not significantly different ($p<0.05$, Duncan's multiple range test).

Ascorbic acid analysis

Ascorbic acid extraction was performed according to the method of Wimalasiri and Wills (1983). After stem and seed removal, pepper samples (5g) were homogenized in 25 mL of 3% citric acid and filtered through miracloth. Sep-Pak C_{18} cartridges were used to remove interfering compounds prior to HPLC. Pepper extract (4 mL) was passed through Sep-Pak C_{18} cartridges which were preconditioned with methanol (4 mL) and water (10 mL). The first 3 mL was discarded and next 1 mL was used for analysis. HPLC analysis was performed using a Bondapak-NH, column (4 mm × 30 cm; Waters Associates, Milford, MA) with a mobile phase of acetonitrile:water (70:30) with 0.01M ammonium di-hydrogen phosphate (pH 4.3) at 2 mL/min.L - ascorbic acid was detected at 254 nm. Recovery rate for ascorbic acid was 99%.

Statistical analysis

Values represent means of three replications, with a pepper fruit serving as a replication. Data were analyzed by analysis of variance ($p < 0.05$), and means separated by Duncan's multiple range test (SAS Institute Inc., 1985).

RESULTS & DISCUSSION

FOR EXTRACTION AND ANALYSIS of pepper flavonoids, a 50% methanol extraction was more effective than 20% or 80%. In pepper extracts, luteolin and quercetin conjugates behaved differently in 1N and 2N HCl/50% MeOH solutions (Fig. 1). Luteolin conjugate was more resistant to acid hydrolysis than quercetin conjugate. Therefore, a 30 min hydrolysis with 1N HCl in 50% MeOH at 90°C was used for quercetin determination, and a 2 hr hydrolysis with 2N HCl in 50% MeOH at 90°C was used for luteolin determination. After acid hydrolysis, quercetin and luteolin were separated by HPLC (Fig. 2). Quercetin has a hydroxyl group at C-3 in the aromatic ring while luteolin does not. Hydrolysis of flavonoid glycosides is affected by types of bonding (C- or O-glycosidic), positions that sugar bind and selective removal of sugar (Markham, 1989). Acid hydrolysis is typically used to analyze flavonoid aglycones. Flavonoid extraction may be affected by MeOH concentration and hydrolysis conditions for fruits and vegetables (Hoffman et al., 1983).

In 50% methanol extracts, pepper flavonoid aglycones were barely detectable (data not shown). After acid hydrolysis, two major flavonoids, quercetin (a flavonol), and luteolin (a flavone), were found. In mature fresh green or yellow stages of maturity, flavonol and flavone contents varied from nondetectable to 800 mg/kg according to pepper type. "Chile," "yellow wax", "ancho", and "serrano" peppers had high concentrations of both luteolin and quercetin compared with "jalapeño" peppers (Table 1). "Yellow wax" type peppers, cv Hungarian Yellow and Long Hot Yellow, had the highest flavonoid content (851 mg/kg and 550 mg/kg respectively), while the hybrid "yellow wax" pepper, Gold Spike, had lesser amounts. "Chili" cv's, New

Table 2—Total soluble phenolics, Sep-Pak C_{18} bound phenolics and antioxidant activity of fresh pepper fruit[a]

Type	Cultivar	Total Phenolics (mg/100g)	Sep-Pak bound phenolics[b] (mg/100g)	Antioxidant activity (%)[c]
Jalapeno	Veracruz	178.2 f[d]	18.7 g	50.1 g
	Mitla	179.1 f	23.9 fg	59. ef
	Tam Mild	243.7 e	25.5 fg	51.5 fg
	Jaloro	348.7 bc	30.2 ef	62.7 ed
	Sweet Jalapeno	244.3 e	37.6 de	67 ed
Wax	Hungarian Yellow	384.9 a	84.9 a	79.5 ab
	Long Hot Yellow	354.3 ab	73.7 b	81.1 a
	Gold Spike (Hybrid)	381.2 a	54.9 c	70 cd
Chile	New Mexico-6	253.9 e	40.0 d	71.6 bcd
	Green Chile	320.3 cd	50.7 c	76.6 abc
Ancho	San Luis Ancho	305.1 d	58.2 c	81.5 a
Serrano	Hidalgo	295.5 d	71.2 b	71.5 bcd

[a] Maturity is green mature stage except yellow type peppers.
[b] Methanol fraction collected from Sep-Pak C-18 cartridges.
[c] Antioxidant activity = % inhibition relative to a control.
[d] Means in each column with similar letters not significantly different ($p<0.05$, Duncan's multiple range test).

Table 3—L-ascorbic acid content of fresh pepper fruit[a]

Types	Cultivar	Ascorbic acid (mg/100g)
Jalapeno	Veracruz	71.6 c[b]
	Mitla	48.9 c
	Tam Mild	65.5 c
	Jaloro	130.8 b
	Sweet Jalapeno	54.0 c
Wax	Hungarian Yellow	114.4 b
	Long Hot Yellow	114.0 b
	Gold Spike (Hybrid)	114.6 b
Chile	New Mexico-6	130.2 b
	Green Chile	121.8 b
Ancho	San Luis Ancho	168.4 a
Serrano	Hidalgo	63.9 c

[a] Maturity is green mature stage except yellow type peppers.
[b] Means in each column with similar letters not significantly different ($p<0.05$, Duncan's multiple range test).

Mexico-6 and Long Green Chili, "ancho" cv San Luis Ancho, and "serrano" cv, Hidalgo had intermediate levels of flavonoids. All "jalapeno" peppers had relatively low flavonoid contents (nondetectable to 53 mg/kg). One exception was the yellow "jalapeño" pepper, Jaloro, which contained 188 mg/kg. All yellow pepper fruit contained high levels of flavonoids. Quercetin content was 2–12 times higher than luteolin in the 12 cultivars and those peppers containing high levels of quercetin typically had high levels of luteolin ($r^2=0.73$).

Major flavonoids in vegetables are the flavonols, quercetin and kaempferol. None of the 28 vegetables and 9 fruits previously tested contained quercetin and luteolin together (Hertog, 1992). In these fruits and vegetables, quercetin content ranged from not detectable to 45 mg/kg, except for onions (347 mg/kg). A few vegetables including celery (22 mg/kg) and red bell pepper (11 mg/kg) contained relatively low levels of luteolin (Hertog et al., 1992). Note that some onions have high reported quercetin levels but the content of this compound has varied greatly among cultivars (Bilyk et al., 1984; Leighton et al., 1992).

In *Capsicum frutescens*, flavonoids were present as both flavonol and flavonone glycosidic conjugates (Sukrasno and Yeoman, 1993). Contents of flavonoids and other phenolics including capsaicinoids vary with fruit maturation. During pepper fruit development, flavonoid synthesis may compete with capsaicinoid synthesis in the phenylpropanoid pathway. The onset of capsaicinoid accumulation in chili pepper fruit was paralleled by the disappearance of flavonoids (Sukrasno and Yeoman, 1993). However, heat index, which represents total capsaicinoid content, was not inversely associated with total flavonoid content in 12 cultivars of peppers tested (Table 1). Moreover, mild peppers did not have greater flavonoid concentrations than hot

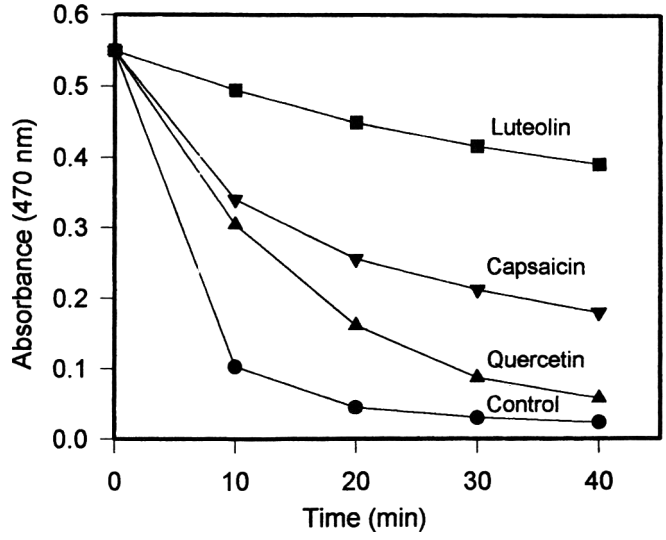

Fig. 3—Antioxidant activity of luteolin, capsaicin and quercetin (0.5 mM each), measured by the bleaching time of β-carotene at 50°C.

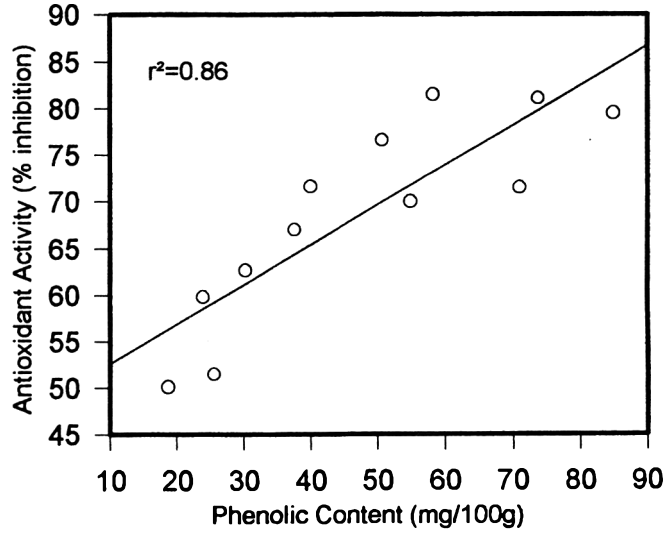

Fig. 4—Relationship between antioxidant activity and lipophilic phenolic content in pepper fruit.

peppers. Biosynthesis of flavonoids may compete with capsaicinoid synthesis in phenylpropanoid metabolism and each pepper type may regulate flavonoid synthesis differently.

Sep-Pak C_{18} bound phenolics accounted for 10–25% of the amount of total phenolics (Table 2). This difference may be due to the polar glycosylated nature of phenolic compounds and/or conditions that influence binding of phenolics to such cartridges. "Yellow Wax" peppers had the highest total and bound phenolic content, "chili", "ancho", and "serrano" peppers were intermediate, and jalapeño peppers had lowest levels. Reverse phase column materials absorb flavonoid glycosides well and total flavonoid content correlated well with Sep-Pak C_{18} bound phenolic content ($r^2=0.88$). Thus major components of Sep-Pak C_{18} bound phenolics were likely flavonoids.

Phenolic compounds including pepper flavonoids can contribute to antioxidant activity (Larson, 1988), as shown by TBA tests and β-carotene bleaching systems (Pratt, 1992). In order to test pepper antioxidant activity, 80% ethanol extracts and Sep-Pak C_{18} cartridges were used to remove interfering polar compounds such as ascorbic acid, which may act as a prooxidant in the presence of metal ions (Niki, 1991). Methanol fractions from

Sep-Pak C_{18} cartridges contained flavonoids, lipophilic phenolics and other non-polar compounds, which may contribute to antioxidant activity in pepper extracts. In a standard chemical assay, luteolin had the highest antioxidant activity when compared with quercetin and capsaicin on an equimolar basis (Fig. 3). Quercetin and luteolin have ortho-dihydroxylation in the aromatic (B) ring which is responsible for major antioxidant activity (Pratt, 1992). Note that C-3 hydroxylation in the flavone structure reduced antioxidant activity. Capsaicin also exhibited good antioxidant activity in the β-carotene bleaching system. Cultivars which had low flavonoid content exhibited antioxidant activity. Residual activity may have been due to the presence of phenolics and capsaicinoids in the ethanol extract, since flavonoid extraction conditions did not result in complete capsaicinoid extraction (Hoffman et al., 1983; Larson, 1988; Reddy and Lokesh, 1992). In general, antioxidant activity correlated well with Sep-Pak C_{18} bound phenolic content in 12 cvs. of peppers (r^2=0.86, Fig. 4). The major antioxidant compounds in the lipophilic fraction isolated from peppers were the flavonoids, quercetin and luteolin. These flavonoid glycosides are hydrolyzed by human bacterial glucosidases and the liberated aglycones have antioxidant activity. Pratt et al. (1992) reported that hydrolyzed fractions had greater antioxidant activity than unhydrolyzed fractions in vegetables that contained flavonoid glycosides.

Ascorbic acid content in peppers varies according to maturity and processing (Howard et al., 1994). Dehydroascorbic acid and L-ascorbic acid are convertible, but, L-ascorbic acid content was much higher than dehydroascorbic acid content in green mature peppers. Among 12 cvs analyzed, L-ascorbic acid concentration ranged from 46.7 mg/100g to 168.3 mg/100g (Table 3). "Yellow wax," "chile", and "ancho" type peppers had more than 100 mg/100g, but "jalapeño" and "serrano" had < 100 mg/100g. An exception was the jaloro variety which contained 130.8 mg/100g. These ascorbic acid concentrations were slightly lower than previous results from similar cultivars (Howard et al., 1994). Differences may be due to experimental or environmental conditions. However, our data confirmed that "chile" peppers had higher ascorbic acid content than "jalapeño" and "serrano" peppers.

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Protein Isolation from Tomato Seed Meal, Extraction Optimization

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ABSTRACT

Water extraction of tomato seed meal proteins was studied to find optimal conditions for protein extraction and isolation. A central composite design including temperature, pH, time and water/solids was used and second order models were employed. Optimum conditions were: 50°C, pH 11.5, 20 min and water/solids = 30/1 (v/w). Experimental values were: extraction yield (extracted protein to that in raw material) 66.1%, protein content of product 72.0%, and total protein yield (protein in isolated product to that in raw material) 43.6%. Estimated values were in good agreement with experimental values. Optimum conditions were confirmed by a larger scale experiment.

Key Words: tomato, protein extraction, seed meal, optimization

INTRODUCTION

ENVIRONMENTAL POLLUTION caused by food processing wastes could be reduced by appropriate recovery of edible nutrients (Birch et al., 1976; Green and Kramer, 1979; Knorr, 1983). Tomato processing wastes, primarily skins and seeds, comprise 10 to 30% of raw fruit weight (Ben-Gera and Kramer, 1969; Geisman, 1981). Tomato seeds represent 50–55% of the pomace. Tomato pomace is mainly disposed of as animal feed or fertilizer (Tsatsaronis and Boskou, 1975; Canella et al., 1979; Cantarelli et al., 1989). A small fraction of the seeds is used by the oil industry (Canella et al., 1979; Geisman, 1981). About 1×10^6 metric tons of tomatoes are processed into products annually in Greece (NSSG, 1990), generating ≈100,000 tons of tomato seeds.

The potential of tomato seeds as a food source has been reported (Ammerman et al., 1963; Drouliskos, 1976; Kramer and Kwee, 1977a,b; Abdel-Rahman, 1982; Al-Wandawi et al., 1985; Lasztity et al., 1986; Rahma et al., 1986). The approximate composition of tomato seeds (dry basis) is: fat 11–20%, protein 15–22% and ash 3–7%. The high unsaturated fatty acid content of tomato seed oil ($C_{18:1}$ 20%, $C_{18:2}$ 55–60%, $C_{18:3}$ 2%) and the nutritive value of the protein compare favorably with soybeans (Rymal, 1973; Rymal et al., 1974; Brodowski and Geisman, 1980; Lazos and Kalathenos, 1988). The high lysine content (8–10 g/16 g N) of tomato seed protein (Rymal et al., 1974; Cantarelli et al., 1989) makes it suitable for supplementing proteins in cereal products (Brodowski and Geisman, 1980; Carlson et al., 1981; Yaseen et al., 1991). In addition the functionality of tomato seed proteins may have many uses in food systems (Kramer and Kwee, 1977a; Moharram et al., 1984; Doxastakis et al., 1988a,b; Doxastakis et al., 1988; Kiosseoglu et al., 1989). Tomato seeds lack antinutritional factors or toxic substances often found in other non-conventional protein sources (Rahma et al., 1986). Thus, the recovery and utilization of tomato seed protein for human consumption has been studied (Kwee, 1970; Canella et al., 1979; Doxastakis et al., 1988b; Cantarelli et al., 1989; Kiosseoglu et al., 1989).

Protein has been isolated from tomato seeds using a 3-step process: extraction, precipitation and drying of protein precipitate (Kramer and Kwee, 1977b; Fazio et al., 1983). Canella and Castriota (1980) examined the effects of several individual factors on protein extraction from tomato seed meal. Latlief and Knorr (1983a,b) studied the protein precipitation step using commercial tomato seeds.

Our objective was to determine the optimal conditions for protein extraction from defatted tomato seed meal, examining simultaneously effects of temperature, pH, time and water-to-solids ratio. The effect of extraction conditions on protein yield and on protein content of isolated product was also determined.

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MATERIALS & METHODS

Materials

Tomato pomace was obtained from a tomato processing plant (KOPAIS S.A. Aliartos, Greece). It was sundried (25–30°C, 3–4 days) and ground in a blender (Waring Commercial Blendor, Dynamics Co., New Hartford, CO). The major part of the skins was removed using a 1 mm sieve. The skins remaining on the sieve were separated from seeds with a fan blowing an upward airstream. The seed fraction was ground (Ultra-Centrifugal Mill, Type ZM1, F.K. Retsch GmbH & Co, Haan, Germany) to pass a 1 mm sieve. Tomato seed meal was prepared by defatting ground seeds with n-hexane in a Soxhlet apparatus and grinding (Ultra-Centrifugal Mill, Type ZM1, F.K. Retsch GmbH & Co, Haan, Germany) to pass a 0.5 mm sieve.

Protein isolation from tomato seed

Tomato seed meal (10 g) was extracted with deionized water (10:1–30:1 ratio) in a stirred glass vessel. The pH of the suspension (7.5–11.5) was kept constant during the extraction by adjusting with 0.5N NaOH. Temperature (30–50°C) was regulated within ±0.2°C by a water bath. The slurry was centrifuged at 2600×*g* for 20 min, the supernatant was collected and the pH was adjusted to the isoelectric point (3.9) using 0.5N HCl. The protein precipitate was separated by centrifugation at 2600×*g* for 25 min and freeze dried. The solid residue after protein extraction was dried at 60°C under vacuum and was used for protein determination.

Isoelectric point (pI)

The pI of tomato seed proteins was determined as the pH value of maximal precipitation. 20g of tomato seed meal was extracted as described, under the conditions: water-to-solids ratio 20:1 (v/w), pH 10, 40°C, 30 min. The pI was found by titrating aliquots of the collected extract to specific pH values and determining the protein content of the supernatant after centrifugation. The protein content was determined according to the method of Lowry et al. (1951).

Analytical methods

Moisture, crude fat, ash, total dietary fiber and crude protein (Nx6.25) were determined according to standard methods (AOAC, 1990). Minerals were determined by atomic absorption/emission spectroscopy (Perkin-Elmer Model 2380, Perkin Elmer Co., Norwalk, CT). Phosphorus was determined photometrically by the ascorbic acid method (Hach Company, 1989), after digestion with concentrated sulfuric acid and hydrogen peroxide (50%) in a Digesdahl apparatus (Hach Company, Loveland, CO). Total sugars were measured according to the phenol-sulfuric acid method (Dubois et al., 1956) using glucose as standard.

Table 1—Variables and levels for central composite design

Variable	Symbol	Coded variables levels[a] −2	−1	0	1	2
Temperature (°C)	x_1	30	35	40	45	50
pH	x_2	7.5	8.5	9.5	10.5	11.5
Time (min)	x_3	20	30	40	50	60
Water-to-solids ratio (v/w)	x_4	10:1	15:1	20:1	25:1	30:1

[a] Passage from coded variable (X_i) level to natural variable (x_i) level is given by the following equations: $x_1=5X_1 + 40$; $x_2=X_2 + 9.5$; $x_3=10X_3 + 40$; $x_4=(5X_4 + 20):1$.

Table 2—Proximate composition and mineral content of tomato seed meal (dry basis)

	Tomato seed	Tomato seed meal
Moisture (%)	8.3	8.1
Protein (%)	25.5	31.3
Crude fat (%)	18.2	1.3
Ash (%)	3.7	4.6
Total sugars (%)	2.9	3.2
Total dietary fiber (%)		54.1
K (mg/100 g)		1046
Na (mg/100 g)		70
Ca (mg/100 g)		294
Mg (mg/100 g)		491
P (mg/100 g)		903
Fe (mg/100 g)		10
Mn (mg/100 g)		6
Cu (mg/100 g)		2
Zn (mg/100 g)		4

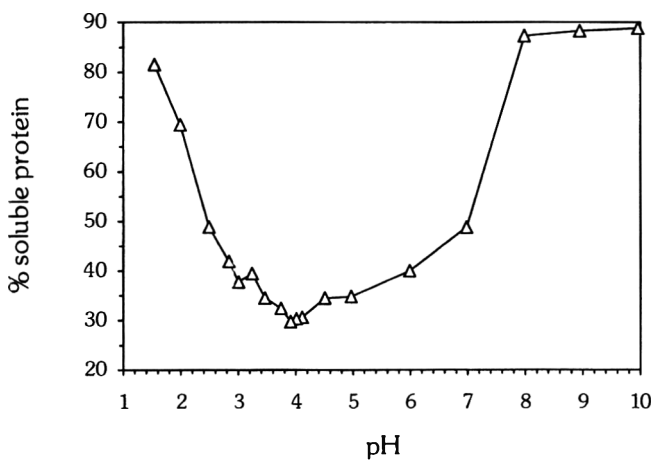

Fig. 1—Precipitation of extracted proteins from tomato seed meal as related to pH.

Experimental design and statistical analysis

The process variables (factors) and the responses (dependent variables) were defined from preliminary studies and published data (Latlief and Knorr, 1983a; Rustom et al., 1991). The process variables (x_i) were: temperature (x_1), pH (x_2), and time of extraction (x_3) and water-to-solids ratio (x_4). Each variable was coded at five levels: −2, −1, 0, 1, 2 (Table 1).

Selected responses which evaluate the extraction process included protein extraction yield (EY) defined as the ratio of total extracted protein to total protein in the raw material, expressed as percentage. Also included were protein content of the product (PR) and total protein yield (TY) defined as the ratio of total protein in the isolated product to total protein in the raw material, expressed as percentage.

A central composite design (CCD) was arranged to allow for fitting of a second-order model (Cohran and Cox, 1957; Adler et al., 1975). The CCD combined the vertices of a hypercube whose coordinates were given by the 2^n factorial design (runs 1–16) with the "star" points (runs 17–24). The star points were added to the factorial design to provide for estimation of curvature of the model (Joklegar and May, 1987). Seven replicates at the center point of the design (runs 25–31) were used to allow for estimation of the "pure error" sum of squares. All experiments were carried out in a randomized order to minimize any effects of extraneous factors on the observed responses. The regression coefficients and the ANOVA tables were computed using the Data analysis-Regression option of EXCEL 5.0 (Microsoft Corporation) program.

Table 3—Central composite design arrangement and responses (1–31) and experimental runs at the optimum conditions (32–39)

Run	Variable levels[a] X_1	X_2	X_3	X_4	Responses EY	PR	TY
1	1	1	1	1	56.60	73.38	37.64
2	1	−1	1	1	49.91	67.66	26.16
3	−1	1	1	1	57.31	74.90	38.87
4	−1	−1	1	1	48.53	71.34	34.62
5	1	1	−1	1	57.16	72.58	40.98
6	1	−1	−1	1	49.98	70.60	30.84
7	−1	1	−1	1	53.06	72.39	34.84
8	−1	−1	−1	1	50.02	69.77	29.57
9	1	1	1	−1	48.74	74.64	30.55
10	1	−1	1	−1	43.91	73.50	30.82
11	−1	1	1	−1	50.60	76.22	37.84
12	−1	−1	1	−1	48.11	72.04	30.43
13	1	1	−1	−1	54.02	74.95	28.77
14	1	−1	−1	−1	50.14	74.48	26.17
15	−1	1	−1	−1	48.30	70.35	29.03
16	−1	−1	−1	−1	46.49	71.75	32.12
17	2	0	0	0	46.63	73.94	24.48
18	−2	0	0	0	51.45	69.37	30.75
19	0	2	0	0	55.41	69.83	35.96
20	0	−2	0	0	48.37	69.32	26.95
21	0	0	2	0	49.02	75.23	36.24
22	0	0	−2	0	51.71	74.36	29.37
23	0	0	0	2	53.32	71.56	35.83
24	0	0	0	−2	42.83	72.64	24.90
25	0	0	0	0	54.49	71.97	35.38
26	0	0	0	0	51.35	75.00	33.35
27	0	0	0	0	50.20	74.05	31.90
28	0	0	0	0	47.85	74.34	29.80
29	0	0	0	0	51.38	73.24	34.41
30	0	0	0	0	46.07	74.48	29.35
31	0	0	0	0	48.13	72.18	30.44
32	−2	2	2	2	63.67	71.79	41.05
33	−2	2	2	2	65.62	71.81	39.93
34	−2	2	2	2	62.51	72.45	42.46
35	−2	2	2	2	64.29	72.07	40.28
36	2	2	−2	2	67.23	69.16	40.07
37	2	2	−2	2	67.73	71.92	42.82
38	2	2	−2	2	65.19	73.37	46.13
39	2	2	−2	2	64.19	73.42	45.31

[a] Coded variables

Optimization

Optimum extraction conditions were estimated by the steepest ascent method (Adler et al., 1975) using a computer program written in BASIC. The program using the fitted model for each response searched the experimental space for optimum responses that were generated by feasible combinations of all factors simultaneously. Several experimental runs were conducted at the predicted optimum conditions; these runs in combination with the earlier ones were used for estimation of new coefficients for fitted models.

Contour plots

Variables with significant linear terms were chosen for axes of contour plots for each response. Contour plots were generated by assigning constant (zero) values to two of the four variables and solving the fitted equations as a quadratic equation in the remaining two variables.

RESULTS & DISCUSSION

Dried tomato pomace consisted of about 53% seeds and 47% skins (weight basis). The seeds were separated from skins, not only because they contained most of the proteins but also because the essential amino acid content and the biological value of seed proteins are higher than those of skin proteins (Lasztity et al., 1986).

The seeds had a protein content of 25.5% (dry basis) considered adequate for protein recovery. Seeds were defatted, since tomato seed oil is recognized as an edible oil (Canella et al., 1979). The proximate composition of tomato seeds and tomato

Table 4—Regression coefficients for the fitted second-order models

Coefficients	Initial models			New models		
	EY	PR	TY	EY	PR	TY
b_0	49.924	73.609	32.090	50.295	73.860	32.792
b_1	−0.067	0.507*	−1.164*	−0.02[illegible]	0.590**	−1.154**
b_2	2.199****	0.804***	2.325****	2.12[illegible]****	0.755**	2.189****
b_3	−0.452	0.356	1.181**	−0.49[illegible]	0.274	1.171**
b_4	2.218****	−0.728**	2.069***	2.146****	−0.777***	1.932****
b_{11}	−0.061	−0.405	−0.803	−0.189	−0.492*	−1.047**
b_{22}	0.652	−0.925***	0.157	0.523	−1.012****	−0.087
b_{33}	0.271	0.380	0.494	0.142	0.293	0.250
b_{44}	−0.302	−0.293	−0.116	−0.431	−0.380	−0.360
b_{12}	0.404	0.022	0.632	0.541	0.270	0.662
b_{13}	−1.176*	−0.854**	−1.112	−0.960**	−0.708**	−0.702
b_{14}	0.089	−0.712**	0.677	0.226	−0.464	0.707
b_{23}	0.430	0.683*	0.497	0.293	0.435	0.467
b_{24}	0.793	0.593*	1.531**	0.576	0.447	1.121**
b_{34}	0.608	−0.183	−0.781	0.471	−0.431	−0.711

* P<0.1
** P<0.05
*** P<0.01
**** P<0.001

Table 5—F values, coefficient of determination and coefficient of variation for the fit of experimental data to models

	Initial models			New models		
	EY	PR	TY	EY	PR	TY
F values	3.91***	4.41***	3.97***	27.15****	3.90***	14.75****
R^2	0.774	0.795	0.778	0.941	0.695	0.896
CV(%)	4.48	1.80	8.55	3.94	1.90	7.23

*** P<0.01
**** P<0.001

Table 6—Observed and predicted responses at the optimum conditions

	EY		PR		TY	
Point	Predicted	Observed[a]	Predicted	Observed[a]	Predicted	Observed[a]
−2,2,2,2	64.2	64.0	72.2	72.0	41.2	40.9
2,2,−2,2	66.1	66.1	71.9	72.0	45.6	43.6

[a] Mean values of four replicates

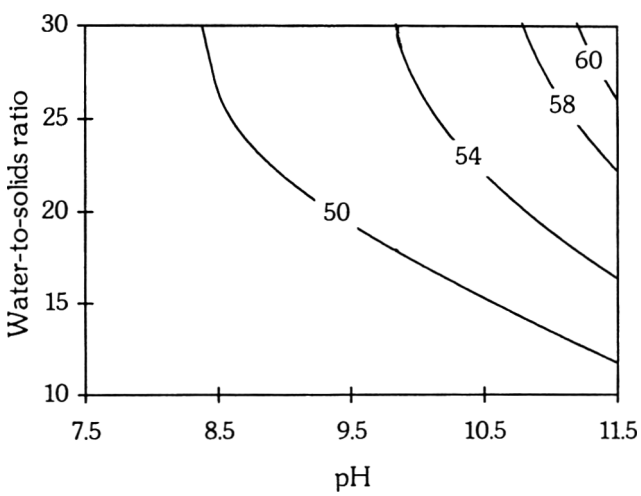

Fig. 2—Contour plots for EY as related to pH and water-to-solids ratio; the other two variables were fixed at zero coded levels.

seed meal were compared (Table 2). Protein content of tomato seeds was higher but ash and crude fat content were slightly lower than reported values (Brodowski and Geisman, 1980; Latlief and Knorr, 1983a; Moharram et al., 1984). This may be due to differences in tomato cultivars and processes.

Tomato seed meal was rich in protein (31.3% d.b.) and compared favorably with other oilseed meals as a potential nonconventional source of protein (Yazicioglu et al., 1981; Liadakis et al., 1993). According to Cantarelli et al., (1989), tomato seed meal is more suitable for protein isolates production because of high fiber content.

Protein precipitation was done at the pI which was 3.9 (Fig. 1). Other values of tomato seed proteins pI have been reported to be between 3.8 and 4.6 (Kramer and Kwee, 1977b; Canella and Castriota, 1980; Latlief and Knorr, 1983a,b; Fazio et al., 1983).

Protein extraction yield (EY), protein content of the product (PR) and total protein yield (TY) obtained by different combinations of the extraction conditions were compared (Table 3). Widely dispersed values of EY were obtained with different combinations of extraction conditions, varying from 42.8 to 57.3%. TY also showed widely dispersed values, from 24.5 to 41%. As protein extraction increased, more proteins were in solution, and more proteins could be precipitated, contributing to total yield increase, thus explaining the same pattern of EY and TY values. However, PR of isolated products did not vary much, ranging from 67.7 to 76.2%, relatively high for such products (Kramer and Kwee, 1977b; Tchorbanov et al., 1986). Nevertheless, these products were characterized as concentrates.

Model fitting

Regression coefficients for the fitted models were compared (Table 4). pH and water-to-solids ratio were the most significant factors for all models. Especially, for the PR model, pH showed highly significant ($P < 0.01$) linear and quadratic terms. This implies that pH and water-to-solids ratio were the predominant factors for modeling of protein isolation from tomato seed meal, as reported by Rustom et al., (1991) for peanuts. Note that temperature was not a significant factor in any models; Canella and Castriota, (1980) reported a similar effect, however, others have reported that temperature influenced protein extraction from proteinaceous sources (Drawert et al., 1979; Rustom et al., 1991).

The adequacy of each model was tested by the lack of fit test and the coefficient of determination R^2 (Table 5). All three models were significant by the F-test at the 1% confidence level and none of the models exhibited lack of fit. Our three models for EY, PR and TY showed R^2 values of 77.4, 79.5 and 77.6% respectively, adequate for models of this type. Reproducibilities of our models were very good (CV 4.5, 1.8 and 8.6% respectively).

Optimization

Fitted models were introduced as objective functions in an optimization program based on the steepest ascent method. Predicted optimum EY values were 70.3 and 67.5 at (−2,2,2,2) and (2,2,−2,2) respectively (variables in coded levels). For PR optimum values of 80.4 and 75.0 were predicted at (2,−0.9,−2,−2) and (−2,1.7,2,2) respectively. For TY the predicted optimum values were 52.0 and 50.5 at (2,2,−2,2) and (−0.5,2,2,2) respectively. As seen from these values, both EY

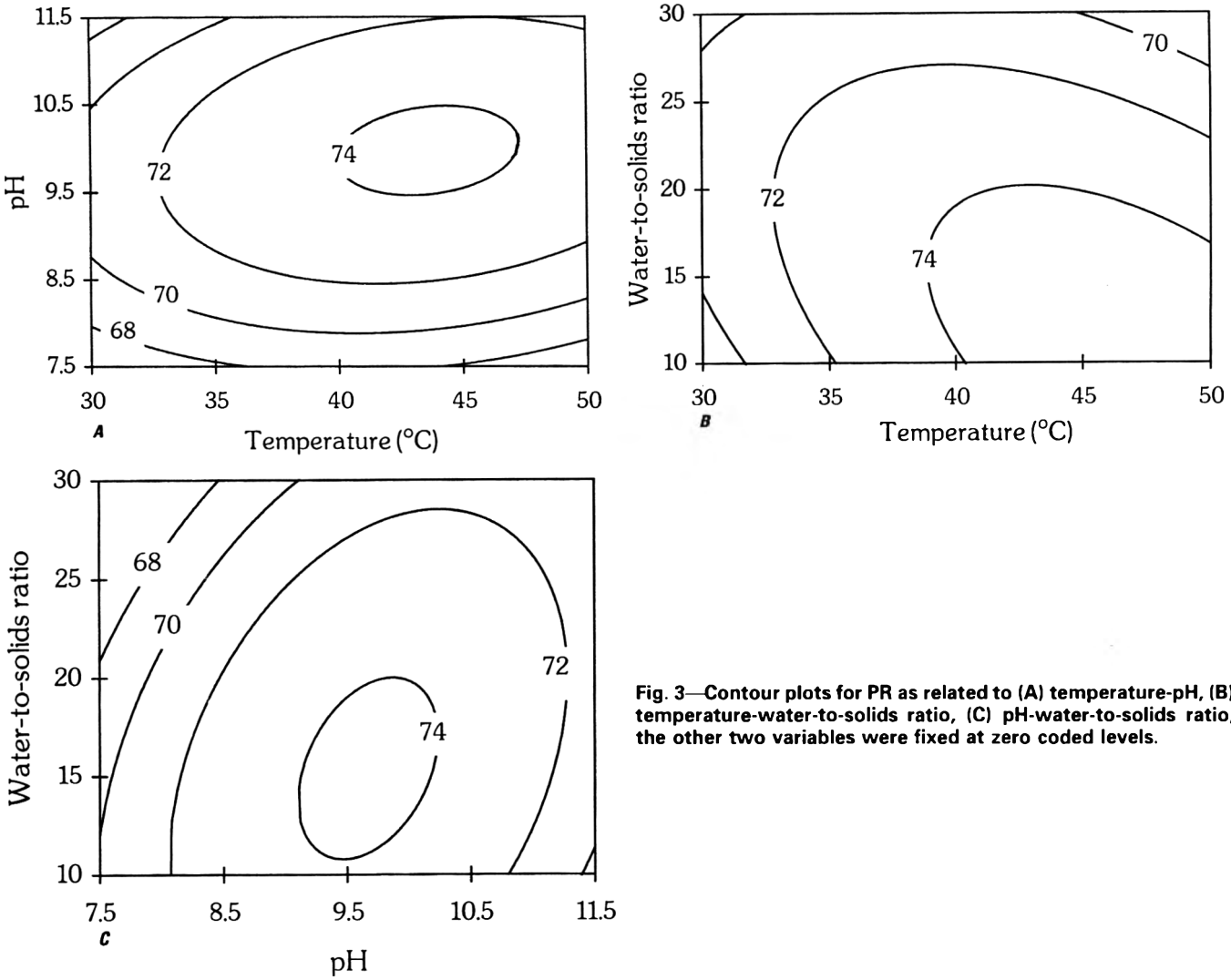

Fig. 3—Contour plots for PR as related to (A) temperature-pH, (B) temperature-water-to-solids ratio, (C) pH-water-to-solids ratio; the other two variables were fixed at zero coded levels.

and TY showed their optimum values at the same factor region. Because protein extraction is the predominant step for protein isolation, we decided to examine further the optimum conditions for EY. Observing optimum EY conditions, note that maximum EY values were obtained at the high level of pH and water-to-solids ratio, low temperature-high time level as well as high temperature-low time level. As is well known, protein solubility increases as pH increases above 7.5 (Kwee, 1970; Kramer and Kwee, 1977b; Canella and Castriota, 1980; Latlief and Knorr, 1983a; Fazio et al., 1983). In addition increased water-to-solids ratio facilitates protein extraction. The combinations of high temperature-short time as well as low temperature-long time give better results of protein extraction, avoiding protein denaturation.

In order to verify the predicted values a series of experimental runs at the optimum conditions was conducted, at the points (−2,2,2,2) and (2,2,−2,2) (Table 3). The observed value for EY compared favorably with the predicted one only at (2,2,−2,2). Because of the discrepancies found between predicted and observed values, we decided to reevaluate the coefficients of fitted equations using all 39 experimental runs (1-31 CCD runs + 32-39 optimum points runs) (Table 4). For all models no essential change was observed for significant factors and interactions; coefficients had slightly modified values, keeping the same sign. The refitted equations were also adequate. All adequacy tests were improved for EY and TY models, especially R^2 being 94.1 and 89.6% for EY and TY respectively. Only PR model showed a slight decrease in R^2 (Table 5).

Predicted values from the refitted equations (Table 6) and observed values were in very good agreement. The (2,2,−2,2) point was selected as conditions giving the maximum EY and TY values.

Contour plots

Variables with significant effect were chosen as axes for contour plots for each response; the other variables were fixed at the central (zero) level. (Fig. 2–4).

EY increased with increasing water-to-solids ratio and pH level (Fig. 2). The highest EY resulted at high pH and water-to-solids ratio. PR showed maximum values at temperature >40°C (Fig. 3A,B), pH between 9.5–10.5 (Fig. 3A,C) and low water-to-solids ratio (Fig. 3B,C). TY increased as pH (Fig. 4A,C) and water-to-solids ratio (Fig. 4B,C) increased. A significant interaction between pH and water-to-solids ratio was observed for TY (Fig. 4C).

A larger scale experiment was conducted at optimum conditions (2,2,−2,2). Tomato seed meal (130 g) was treated following the same procedure. The experiment gave results in close agreement with those of the small scale, with an EY of 64.1%, and a TY of 41.2%. The product proximate composition and the solid residue remaining after protein extraction were analyzed (Table 7). The economic feasibility of protein isolation process also depends on utilization of by-products. The whey remaining after protein precipitation could be concentrated or

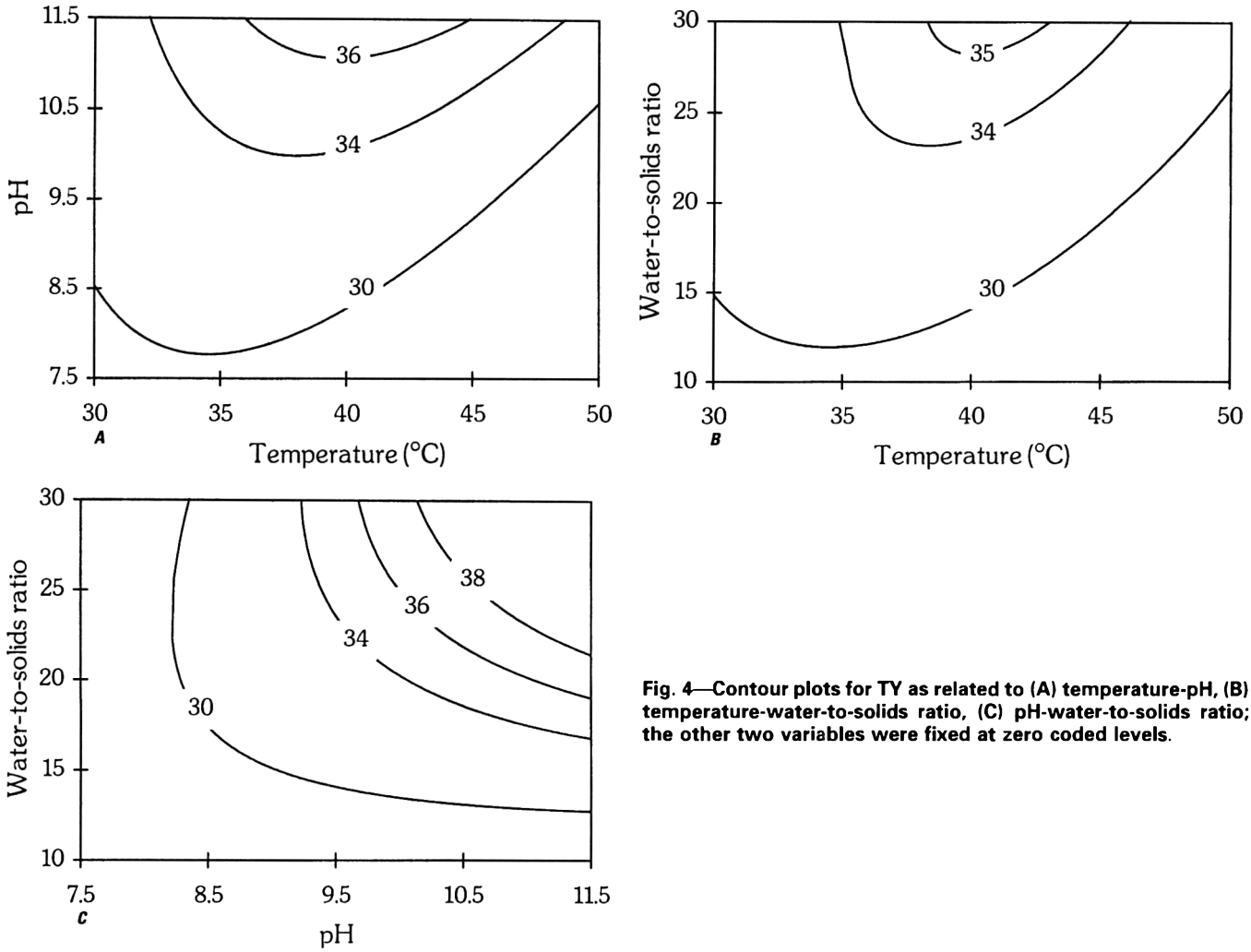

Fig. 4—Contour plots for TY as related to (A) temperature-pH, (B) temperature-water-to-solids ratio, (C) pH-water-to-solids ratio; the other two variables were fixed at zero coded levels.

Table 7—Proximate composition and mineral content of tomato seed protein concentrate and solid residue after protein extraction (dry basis)

	Tomato seed protein conc	Solid residue
Crude protein (%)	71.3	18.5
Ash (%)	3.4	5.5
Total sugars (%)	0.8	0.7
Total dietary fiber (%)	16.1	71.0
K (mg/100 g)	193	400
Na (mg/100 g)	596	912
Ca (mg/100g)	525	214
Mg (mg/100 g)	102	282
P (mg/100 g)	570	473
Fe (mg/100 g)	15	13
Mn (mg/100 g)	2	6
Cu (mg/100 g)	2	1
Zn (mg/100 g)	3	5

spray-dried to recover proteins and the solid residue of the extraction could be used as animal feed.

CONCLUSION

OPTIMUM EXTRACTION of tomato seed proteins with water for protein isolation, could be achieved by extracting one part of tomato seed meal with 30 parts of water (w/v ratio) at pH 11.5 at 50°C for 20 min. These conditions resulted in extracting 66.1% of the proteins contained in tomato seed meal, at a total protein yield of 43.6%. Isolated product had a protein content of 72%.

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Physical Properties of Soy Bean and Broad Bean 11S Globulin Gels Formed by Transglutaminase Reaction

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ABSTRACT

Physical properties of transglutaminase-induced glycinin and legumin gels were compared with thermally induced gels. Results of deformation tests showed that transglutaminase-induced gels were more rigid and elastic than thermally induced gels. From creep compliance tests, all elastic moduli and viscosities except Newtonian viscosity were higher for transglutaminase-induced gels. Electron micrographs revealed that network structures of transglutaminase-induced gels were composed of larger unit particles forming more developed strands and clusters. More rigid and elastic gels were formed from glycinin as compared to legumin by both gelling methods.

Key Words: beans; globulin gels; transglutaminase; thermal gelling

INTRODUCTION

THE GELLING ABILITY of proteins, and physical properties of protein gels are important for use as texture modifiers in foods. Extensive studies have been reported to understand mechanisms of thermal gelation of food proteins (Hermansson, 1978; Saio and Watanabe, 1978; Kinsella, 1979; Clark et al., 1981; Mori et al., 1986). The physical properties of heat-set proteins gels by deformation tests have been also reported by many (Saio et al., 1969; Richardson and Ross-Murphy, 1981; Hermansson and Lucisano, 1982; Stading and Hermansson, 1991).

The range of protein functionality should be broadened as food grade protein cost increases. Therefore, structures of proteins have been modified to create new and unique products that would have improved functional properties in food systems. Enzyme modification successfully improved functional properties (Feeney and Whitaker, 1982). Many reports concern the useful potential of exogenous transglutaminase (EC 2.3.3.13) (TG) in catalyzing cross-linking of food proteins (Ikura et al., 1980; Motoki et al., 1984; Nio et al., 1986; Aboumahmoud and Savello, 1990; Traore and Meunia, 1992; Matsumura et al., 1993; Kang et al., 1994). This enzyme can mediate ε-(γ-glutamyl)lysyl cross-links between food proteins (Folk and Chung, 1973). Endogenous TG may also induce the setting of fish meat sol to gel at room temperature (Seki et al., 1990; Tsukamasa et al., 1993). The unique elasticity of the set gel of fish meat was hypothesized to be due to formation of ε-(γ-glutamyl)lysyl cross-links in gels. No report has established whether the rheological properties associated with TG-related setting of fish gels may be observed in other protein gels. Fundamental information and/or commercial applications may develop from comparing physical properties between conventional thermally induced protein gels and TG-induced ones. Our previous study showed the minimum concentration required to form a self-supporting gel of 11S globulins catalyzed by TG was lower than the minimum concentration for thermal gelation (Chanyongvorakul et al., 1994). This indicated that the covalent bonding by TG was more effective in forming a self-supporting network.

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Analysis and evaluation of food texture can be classified into tests outside of, or within the linear viscoelastic range. Rheological tests outside the linear range, i.e. "large deformation" measurement, has produced valuable correlations with sensory textural evaluations (Bourne, 1982). However, testing within the linear range (static or dynamic testing) can provide important data relating to structure.

Our objective was to compare textural properties of 11S globulin gels formed by TG reaction with those from the conventional heating method by analyzing large deformation behavior and creep compliance behavior of both types of gels. The microstructures determined by scanning electron microscope were also compared.

MATERIALS & METHODS

Materials

Transglutaminase (Ca^{2+}-independent) derived from *Streptoverticillium sp.* No. 8112 by the method of Ando et al. (1989) was supplied by Ajinomoto Co., Inc. (Japan). Soybeans (*Glycine max*, var. Tsuru-no-ko) were purchased from Mizuno Seed Co., Ltd (Japan). Broad beans (*Vicia faba*, var. Sanuki Nagasaya) were purchased from Takii Seed Co., Ltd (Japan). The reagents, of analytical reagent grade, were purchased from Wako Pure Chemical Industries, Ltd (Japan) and Nakarai Tesque, Ltd (Japan).

Preparation of 11S globulins

Glycinin was prepared from acetone powder (Mori et al., 1981) according to the method of Thanh et al. (1975). Legumin was isolated from *Vicia faba* meal using the procedure described by Suchkov et al. (1990) except that heat treatment was omitted. Purified 11S globulins were dialyzed in 35 mM potassium phosphate buffer (pH 7.6) containing 0.4M NaCl (μ = 0.5) (reaction buffer) before use. The dialysis was done four times and each time required around 6 hr, by using 100:1, reaction buffer to protein solution.

Preparation of gels

In the case of TG-induced gelation, 12% 11S globulin solution in reaction buffer was mixed with microbial TG solution. TG was added to protein solution at a ratio of 1:50, w/w. To avoid forming air bubbles, mixtures were carefully poured into stainless steel molds (6 mm i.d., 5 mm ht) sealed at the bottom end with paraffin sheets and placed on a steel plate. Tops of molds containing protein solution were covered with paraffin sheets and another steel plate was placed on top. Both ends of the filled molds were tightly fixed between the two steel plates. Samples were then incubated at 37°C for 5 hr, allowed to stand at 20°C for 20 hr, and carefully removed from molds. The following experiments were carried out immediately.

Following thermal gelation, 12% 11S globulin solution in reaction buffer was injected into the stainless steel molds as described above. In addition, the two steel plates were tightly fastened with clips to prevent heat induced leakage. These were heated in a boiling water bath at 100°C for 1 hr and rapidly cooled to room temperature (≈13°C) by immersion in cold water. Based on preliminary experiments, incubation at 100°C for 1 hr was enough for formation of glycinin and legumin gels. Gels were allowed to stand overnight at 20°C, and then removed from molds before subjecting to the following experiments.

Large deformation tests

Uniaxial compression-decompression tests were performed using a Rheoner RE3305 (Yamaden Co., Ltd). A gel specimen was subjected to various preset deformation levels 1, 2.1 and 3.5 mm corresponding to 20%, 42% and 70% of total height of gel, respectively. A polyacetal plunger (30 mm diameter) compressed the gels at the rate of 0.5 mm/s. Force that developed was recorded continuously. The maximum force was evaluated from the height of the chew profile.

A modulus of deformability was calculated from the slope of the linear portion (until 5.5% deformation) of the stress-strain relationship (Mohsenin and Mittal, 1977). It had stress units and could quantify stiffness of gel. Elastic properties of gels were evaluated on compression-decompression curves (Fig. 1). The area under the compression curve is the total work/unit volume while the area under the decompression curve is the recoverable work/unit volume. The irrecoverable work/unit volume was calculated as the difference between total and recoverable work (i.e. hatched area of hysteresis loop). The ratio of irrecoverable work to the total work was then calculated, and used as a measure of deviation from ideal elasticity (Olkku and Sherman, 1979; Kaletunc et al., 1991). All the mechanical tests were performed at 20°C and means were calculated from seven to eight replicates.

Creep compliance tests

Creep behavior under compression was analyzed using a Rheoner RE3305 (Yamaden Co., Ltd) interfaced with a computer (NEC PC9801N-12, Tokyo). A gel was compressed by a polyacetal plunger (30 mm diameter) at cross-head speed of 5 mm/sec. Based on preliminary tests to ensure measurements were made within the linear range, constant force applied to the gels was 10 g. Creep and recovery were measured for 5 min intervals. Results were analyzed using software (CA-3305-16) developed for creep analysis (Yamaden Co., Ltd, Tokyo). This software was developed according to the procedure described by Inokuchi (1955) and Sherman (1966). All measurements were replicated eight times and performed at 20°C.

Scanning electron microscope tests (SEM)

A rectangular gel specimen was cut (1 × 1 × 2 mm) and fixed in 2% glutaraldehyde, 2% tannic acid and 1% osmium tetra oxide, respectively. The sample was then dehydrated by immersion in a series of ethanol mixtures, 50%, 70%, 80%, 90%, 95% and 100% and finally immersed in isoamyl acetate. After dehydration, critical-point drying was done in liquid CO_2 in a pressurized chamber. The dried sample was carefully fractured into small pieces to reveal internal micro-structure. Fragments were mounted on an aluminum SEM stud by a small droplet of graphite paste and coated with platinum. All samples were then examined and photographed using a S-4100 Hitachi scanning electron microscope.

Protein determination

Protein was determined by the method of Lowry et al. (1951).

Statistical analysis

Data were subjected to analysis of variance and Duncan's Multiple range test. Means and standard deviations were calculated and significant differences between gels were determined.

RESULTS

Large deformation studies

Maximum forces generated by deformation of Ca^{2+}-independent TG and heat-induced 11S globulin gels to various deformation levels were compared (Fig. 2). Breakdown of gel structure, resulting in formation of small cracks could be detected at deformation level 70% which could be interpreted as the fracture point of the gel samples (Vliet et al., 1991). The maximum forces for TG-induced gels were higher than those for thermally-induced gels at any deformation level ($p < 0.05$). In glycinin and also legumin gels, the maximum forces for TG-induced gels were twice those for thermally-induced gels.

Maximum force for glycinin gels was higher than that for legumin gels by both gelation methods. Chanyongvorakul et al. (1994) reported that gel hardness of TG-induced glycinin gels

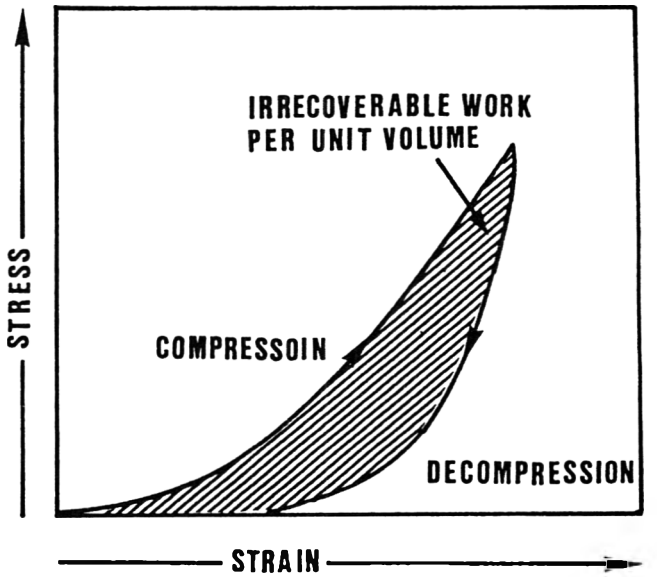

Fig. 1—Schematic of stress-strain relationships in compression-decompression cycle.

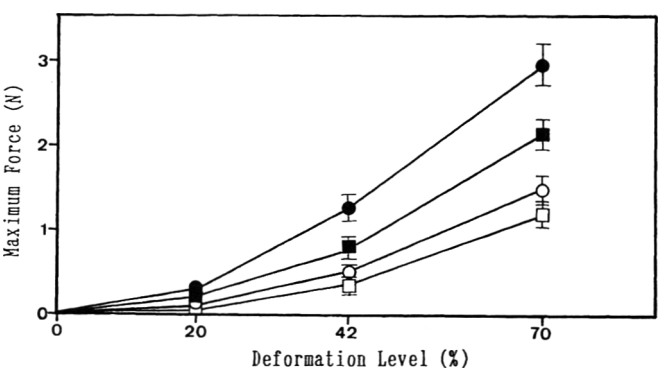

Fig. 2—Maximum force of 11S globulins gels subjected to various deformation levels. (Means with standard deviation bars). ● , transglutaminase-induced glycinin gel; ■ , transglutaminase-induced legumin gel; ○ , thermally induced glycinin gel; □ , thermally induced legumin gel

was greater than that of TG-induced legumin gels at various protein concentrations. For thermally-induced gels, Zheng et al. (1991) clearly showed that glycinin formed stiffer gels in comparison with legumin.

The moduli of deformability of all gels were compared (Table 1) with a deformation level of 42%. The higher values of deformability moduli, indicating stiffer gels, were obtained when TG was used. In both TG- and thermally induced gelation, glycinin gels exhibited larger moduli than legumin gels.

Elastic properties of gels, evaluated as the ratio of irrecoverable work (Fig. 1) to total work were also compared (Fig. 3). Those Data were at deformation levels of 20 and 42%. Irrespective of gelation method, the percentage of irrecoverable work increased substantially with deformation level. This indicates that all gels lost elastic properties upon increasing deformation level. This probably indicates increased damage of gel network structures (Kaletunc et al., 1991). At the same deformation level, the ratio of irrecoverable work to total work of TG-induced gels was lower than those of thermally-induced gels ($p < 0.01$). Note that the ratio of TG-induced gels was <20% even by severe deformation at 42%. This indicated that physical networks of TG-induced gels were more resistant to large deformation than the networks in thermally induced gels.

No difference occurred in elastic properties between glycinin and legumin gels formed by TG reaction at 20% deformation

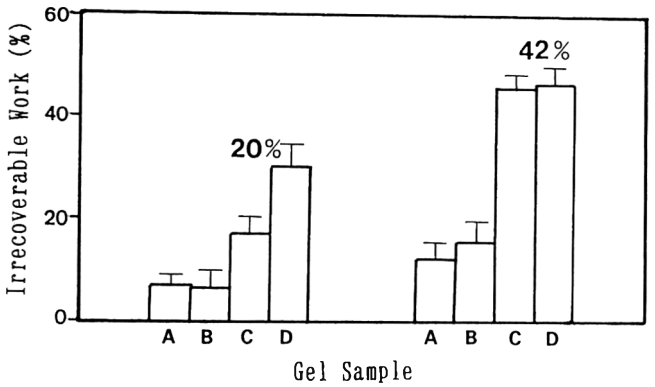

Fig. 3—Ratio of irrecoverable work and total work of 11S globulins gels subjected to 20% and 42% deformation levels. (Means with standard deviation bars). (A) transglutaminase-induced glycinin gel; (B) transglutaminase-induced legumin gel; (C) thermally induced glycinin gel; (D) thermally induced legumin gel.

Table 1—Modulus of deformability for gel samples at 42% deformation

Sample	Modulus of deformability[a] (Pa)
Transglutaminase-induced glycinin gel	$4.4\times10^4 \pm 0.35^b$
Transglutaminase-induced legumin gel	$3.2\times10^4 \pm 0.25^c$
Thermally-induced glycinin gel	$1.5\times10^4 \pm 0.12^d$
Thermally-incuded legumin gel	$0.9\times10^4 \pm 0.12^e$

[a] Modulus of deformability was calculated from slope of the linear portion (until 5.5% deformation) of the stress-strain relationship.
[b-e] Means ± standard deviations with different letters are significantly different by Duncan's Multiple Range test ($p<0.01$).

levels ($p < 0.01$). However, at 42% deformation, the ratio of TG-induced legumin gel was higher than that of TG-induced glycinin gels ($p < 0.05$). In thermally-induced gelation, legumin gels exhibited higher ratios than in glycinin gels at 20% deformation ($p < 0.01$). This indicated that glycinin gel was more elastic.

Creep compliance behavior

Creep and recovery creep compliance curves for TG-induced and thermally-induced gels were compared (Fig. 4). The creep compliance of thermally induced gels was larger than that of TG-induced gels. This implied that the TG-induced gels were stiffer.

Furthermore, recovery behavior was different between TG- and thermally induced gels. The compliance after 550 sec was very large in thermally induced gels, 0.5×10^{-4} m²/N and 0.7×10^{-4} m²/N for glycinin and legumin gels, respectively. For TG-induced gels, on the other hand, compliance was low in both glycinin and legumin gels. No measurable compliance was detected for glycinin. Thus more irrecoverable deformation was found by creep tests on thermally-induced gels than on TG-induced gels. This was consistent with greater loss of elasticity of thermally-induced gels indicated by the large-deformation tests.

Another difference in creep-compliance curves of TG-induced and thermally induced gels was noted in the retarded deformations. In those parts of the curves of thermally induced gels, compliance first increased rapidly followed by gradual increase with time. In TG-induced gels, however, the two phases (rapid and slow increase) were not clearly distinguishable. This difference in the retarded deformation part was also related to creep parameters, particularly retardation time (discussed below).

The rheological behavior of viscoelastic materials within the linear viscoelastic range can be described by several mechanical models consisting of springs and dashpots. In our experiment upon analysis of 11S globulin gel creep curves, a six element Kelvin-Voigt model was fitted into all data. This model was also suited other gels, e.g., whey protein (Katsuta et al., 1990), casein

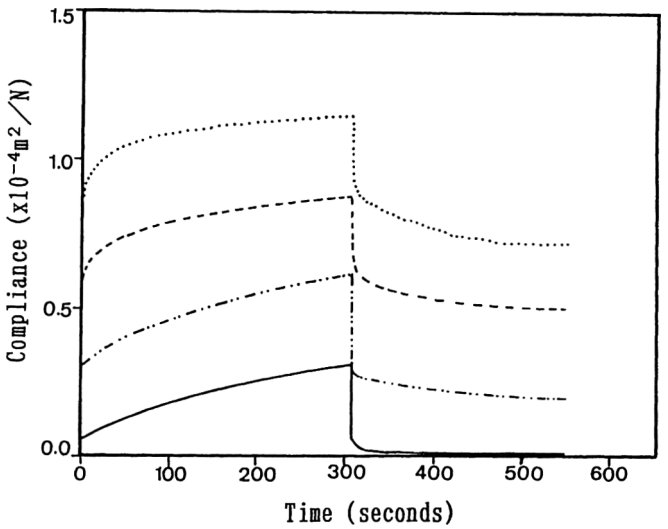

Fig. 4—Creep-compliance curves of various 11S globulins gels. ———, transglutaminase-induced glycinin gel; -·-·-·-, transglutaminase-induced legumin gel; - - - -, thermally induced glycinin gel; ·····, thermally induced legumin gel.

(Halim and Shoemaker, 1990), and soybean protein (Mori et al., 1989).

The creep parameters (E_0, E_1, E_2, η_1, η_2, η_N, τ_{k1}, and τ_{k2}) according to a six-elements model were calculated and compared (Fig. 5). Results between TG-induced and thermally-induced glycinin gels, showed that values of all elastic moduli and viscosities except η_N were higher in TG-induced gels ($p < 0.05$). In legumin gels, E_0, E_2 and η_2 were higher in TG-induced gels than in thermal-induced gels ($p < 0.05$). However, η_N of thermally-induced legumin gels were remarkably higher than TG-induced legumin gels ($p < 0.01$). Obviously, from these data, TG- and thermally-induced gels could be categorized into two different groups. While TG-induced gels were characterized by higher elastic and viscoelastic properties, thermally induced gels had higher values of η_N relative to other elastic moduli and viscosities.

Retardation times of TG-induced gels were longer than those of thermally induced gels ($p < 0.05$). Longer and shorter retardation times meant slower and more rapid deformation of the viscoelastic body, respectively. Shorter retardation times of thermally induced gels corresponded to creep-compliance curves (Fig. 4), in which rapidly increasing phases were clearly observed in the retardation part.

Differences in creep viscoelastic parameters occurred between glycinin and legumin gels formed by TG reaction ($p < 0.05$). All elastic moduli and viscosities of glycinin gels were higher than those of legumin gels. In thermally induced gels, differences occurred in only η_N which was very high for legumin gels ($p < 0.01$). However, E_0, E_2, and η_2 of thermally induced legumin gels were lower than those of thermally induced glycinin gels.

Microstructure

In general, the effects of TG reaction on gels can be observed by unaided eye. Opaque legumin gels were formed by Ca^{+2}-independent TG whereas transparent gels were formed by heat treatment. However, glycinin gels formed by both treatments were white-opaque. But the turbidity of TG-induced glycinin gels seemed to be higher.

SEM micrographs of globulin gels were prepared at 30,000× (Fig. 6) and TG-induced glycinin (Fig. 6A) and legumin (6B) gels, were compared. Thermally induced glycinin (6C) and legumin (6D) gels were also compared. Although magnification was the same a difference in particle size occurred in protein

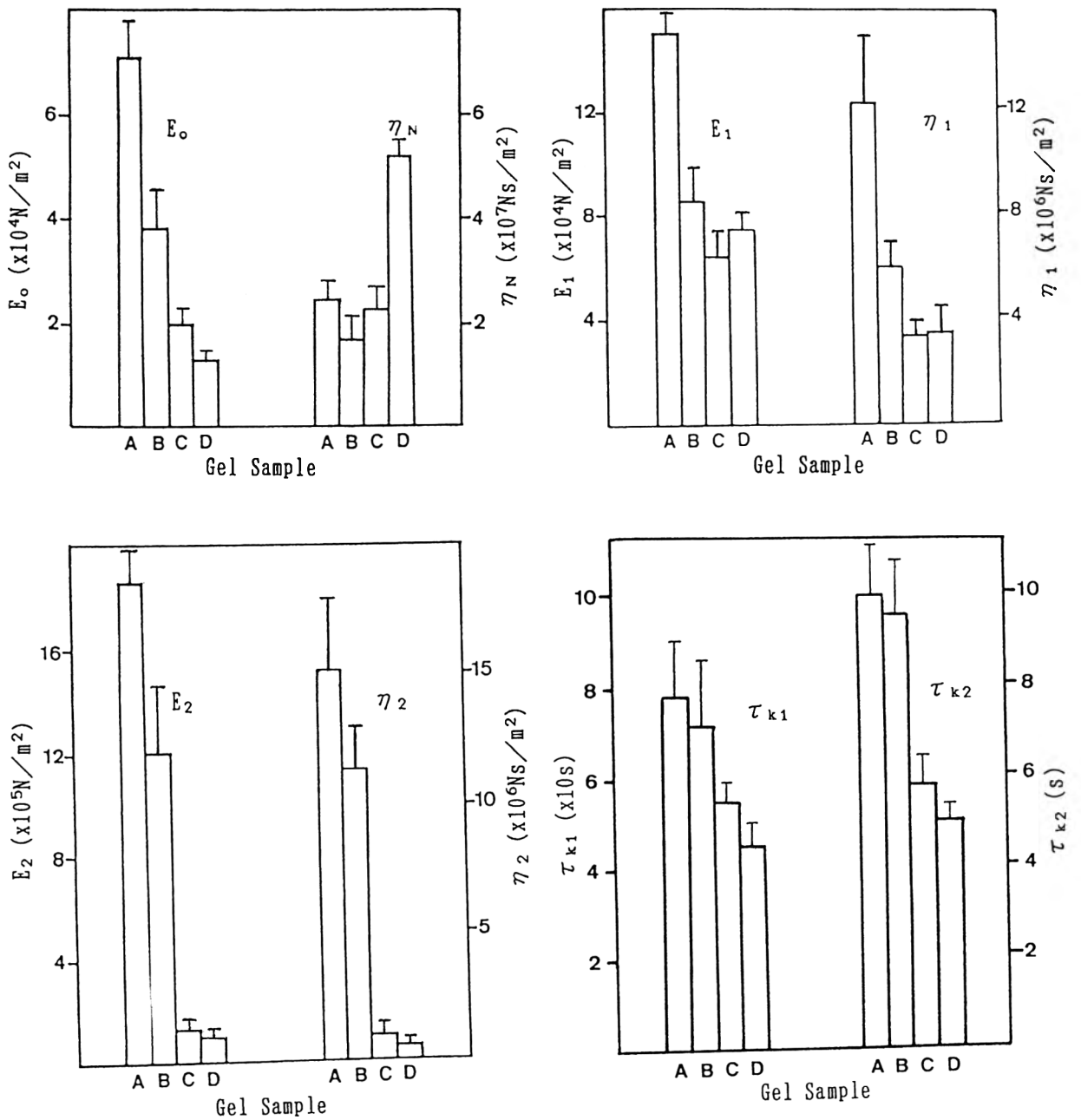

Fig. 5—Viscoelastic parameters in the six element mechanical model of various 11S globulins gels (Means with standard deviation bars). E_0, instantaneous modulus; η_N, final Newtonian viscosity; E_1, retarded elastic modulus and η_1, retarded viscosity of the first retarded compliance; E_2, retarded elastic modulus and η_2, retarded viscosity of the second retarded compliance; τ_1, retardation time of the first retarded compliance; τ_2 retardation time of the second retarded compliance. (A) transglutaminase-induced glycinin gel; (B) transglutaminase-induced legumin gel; (C) thermally induced glycinin gel; (D) thermally induced legumin gel.

aggregates between TG-induced and thermally induced gels. Particularly, notable differences were apparent in legumin gels (Fig. 6B and 6D). Network structure of thermally induced gels was composed of particles of small size forming short and thin strands and small clusters. However, large particles formed strands and big clusters in TG-induced gels. As a result, there was more space among strands or clusters in micrographs of TG-induced gels than in those of thermally induced gels.

In micrographs at 100,000× the network structure appeared more developed in TG-induced gels (Fig. 7A and 7B). Thick strands (≈50 nm in width) form the network structure and clusters more than 100 nm in width were clearly observed in both. In thermally induced gels (Fig. 7C and 7D), particularly legumin gels (Fig. 7D), small particles that were <30 nm in diameter were apparent. The micrographs of both thermally induced gels show that unit particles were smaller and the strands and clusters were less developed than in TG-induced gels.

Microstructures of glycinin and legumin gels formed by TG reaction were not different (A and B of Fig. 6 and 7). However, in thermally induced gels, the microstructure of glycinin gel (Fig. 6C and 7C) and legumin gel (Fig. 6D and 7D) showed some differences. The particles were a little larger than for glycinin gel. The surfaces of glycinin gel particles were not so smooth as those of legumin gels (Fig. 7). In glycinin gels, there was dense association of particles (6C). As a result, more space could be seen indicating effective association of glycinin molecules by heating (6C and 6D).

DISCUSSION

THE CHARACTER OF GELS is very dependant on the nature and/or degree of crosslinking of the physical networks. Previous studies demonstrated that disulfide bonds and non-covalent bonds contribute to formation and stabilization of network struc-

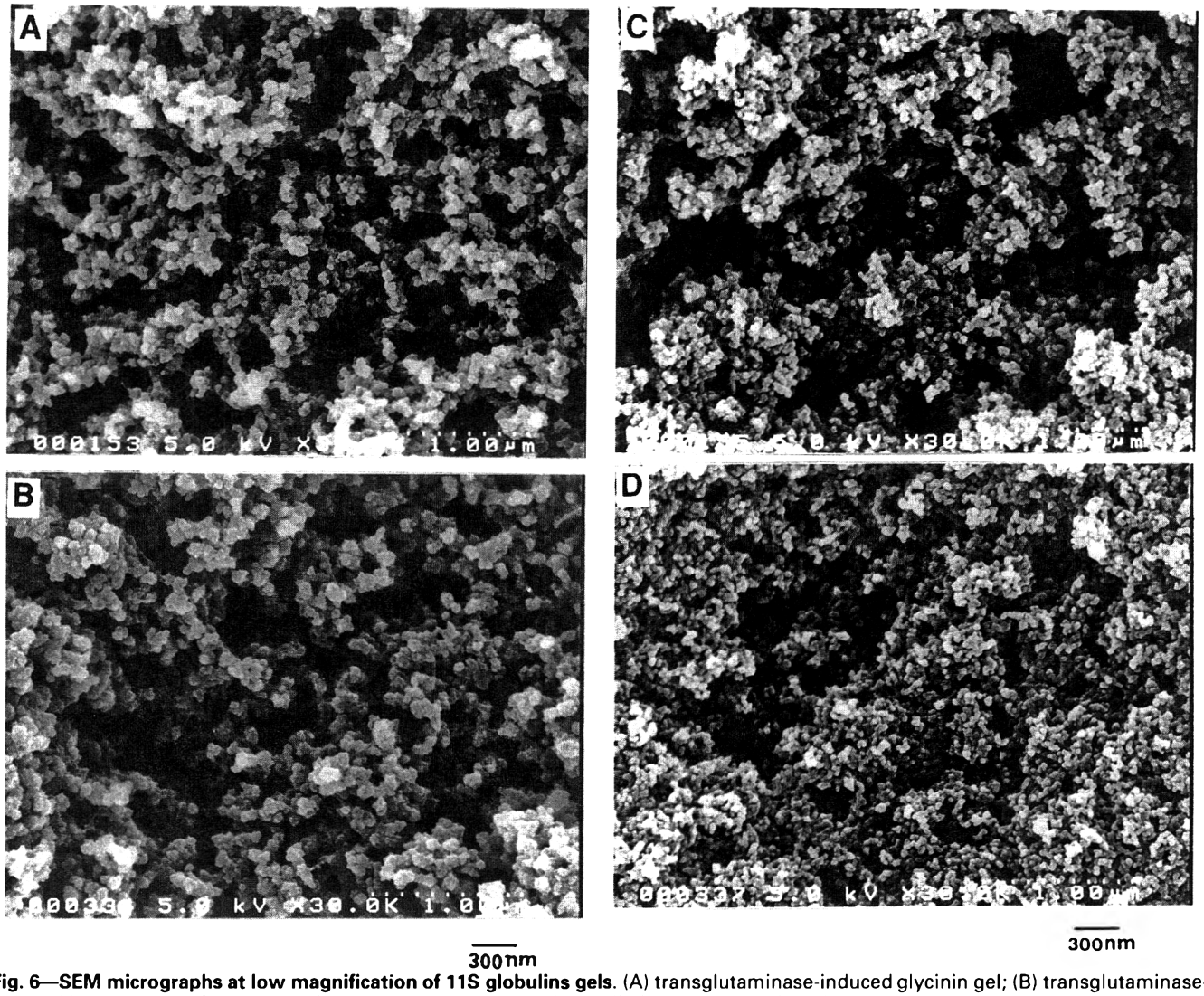

Fig. 6—SEM micrographs at low magnification of 11S globulins gels. (A) transglutaminase-induced glycinin gel; (B) transglutaminase-induced legumin gel; (C) thermally induced glycinin gel; (D) thermally induced legumin gel.

ture of thermally induced globulins gels (Furukawa et al., 1979). When chemical bondings (such as disulfide bond) are involved in crosslinking of protein strands, these bonds may restrict flow of the chains, and enhance elastic properties and/or stiffness. Free sulfhydryl contents of both globulins were low (Draper and Catsimpoolas, 1978; Zheng et al., 1991). Therefore, the formation of disulfide bonds between these protein molecules which would initiate SH-SS interchange reactions, is limited. Thus crosslinks of protein network are stabilized mainly by reversible and weak noncovalent bonds rather than disulfide bonds. This may result in less elastic properties of thermally induced gels.

On the other hand, in the TG-induced gels, another chemical bonding, ε-(γ-glutamyl)lysine isopeptide bond, additionally stabilizes network structures. The crosslinks of this type gel should contain more chemical bondings in comparison with those of thermally induced gels. Chemical crosslinks can restrict the perpetual flow of protein chains effectively. They would develop elastic and viscoelastic parts inside the gel network. Therefore, the TG-induced gels may be more elastic.

Microstructures correlated with data on rheological properties. TG-induced gels had developed network structures formed by thick strands. These may contribute to elastic and stiffness of TG-induced gels. In thermally induced gels, such developed network structure with thick strands was not observed. For thermal gelation, the main region involved in the molecular-molecular interaction should be hydrophobic sites exposed by heat-denaturation. We hypothesize that the exposure and interaction of the hydrophobic region in the protein molecule during thermal denaturation occurs regularly to some extent. However, hydrophobic interactions among molecules may not be so strictly regulated and strong as chemical interactions through covalent bonds. Reaction sites in substrate proteins are strictly recognized by TG (Gorman and Folk, 1980). In the case of TG-induced gelation, therefore, the association of protein molecules through isopeptide bonds may occur more regularly than in thermally-induced gels. Furthermore, the formation of chemical bonds such as isopeptide bonds are irreversible, and may contribute to strong protein-protein interactions. Such associations of protein molecules probably lead to the developed network structure of TG-induced gels demonstrated in our results.

We also found some differences between glycinin and legumin gels formed by the same treatment. These results were consistent with our previous data. Irrespective of gelling method, more rapid formation of soluble aggregates and lower minimum gelling concentration were observed for glycinin gels (Zheng et al., 1991 and Chanyongvorakul et al., 1994). However, a significant homology occurs in the primary structure and a common quarternary structure between glycinin and legumin (Badley et al., 1975). Moreover, the levels of isopeptide bonds in the glycinin and legumin gels formed by the TG reaction were the same (Chanyongvorakul, 1994). Therefore, we hypothesized that the reason for differences in gelling and physical properties of the two globulin gels in previous studies may come from

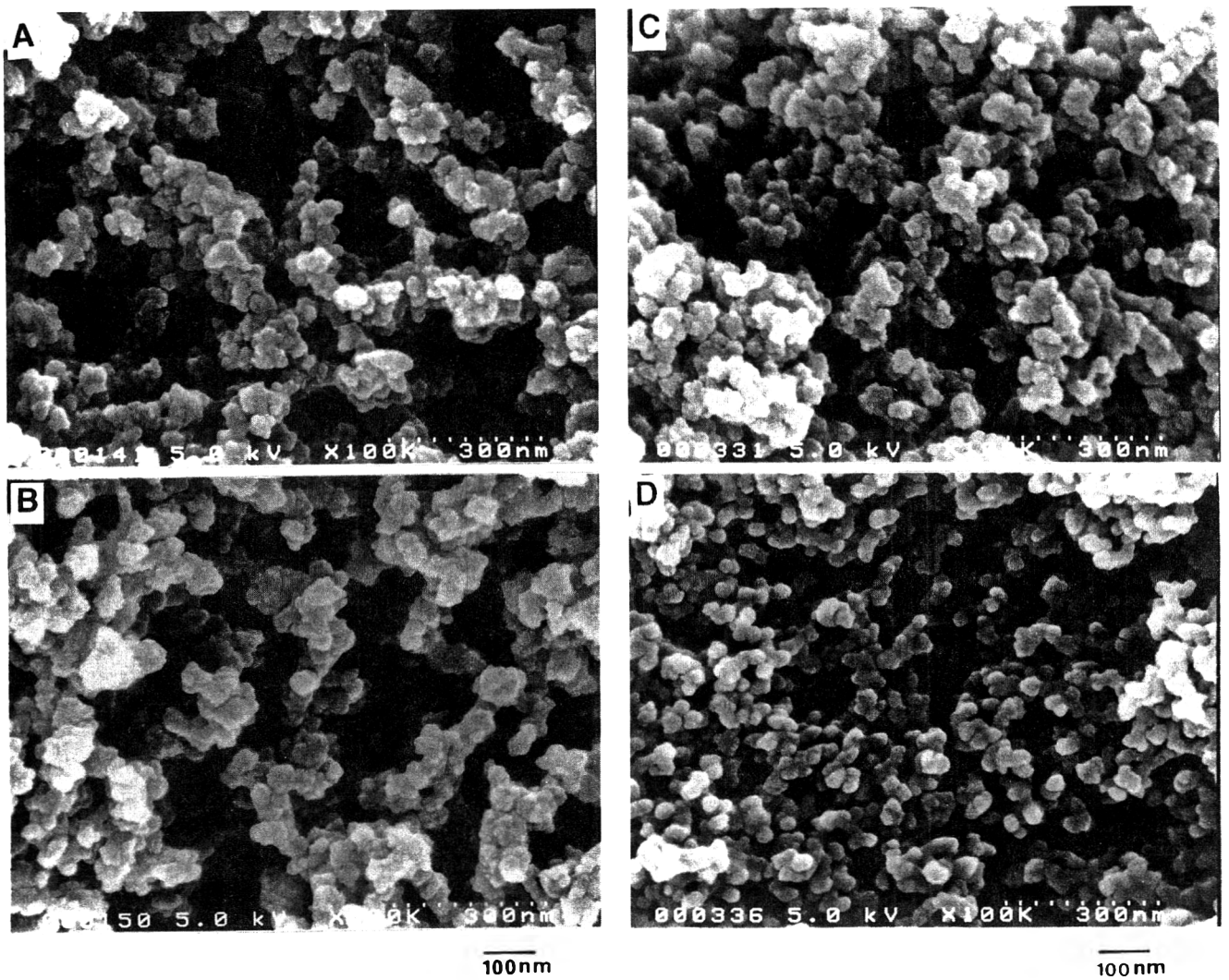

Fig. 7—SEM micrographs at high magnification of 11S globulins gels. (A) transglutaminase-induced glycinin gel; (B) transglutaminase-induced legumin gel; (C) thermally induced glycinin gel; (D) thermally induced legumin gel.

differences in the levels of disulfide and free sulfhydryl residues, and surface hydrophobicity.

In thermal gelation systems, inter-molecular disulfide linkages and non-covalent hydrophobic interactions are the most important in the formation of network structure. These bonds should mainly determine the gelling properties in thermally induced gels. However, no evidence indicated that these bonds were also important for the physical network of TG-induced gels. Further studies including treatments of gels with various reagents destabilizing covalent and/or noncovalent bonds are necessary.

CONCLUSIONS

TG TREATMENT on bean globulins produced stiff gels with high elasticity compared to thermally induced gels. Research is needed on the physical properties of TG-induced gels of other beans, TG treatment could be a new and useful method to modify the texture of food protein gels.

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Flavor and Oxidative Stability of Roasted High Oleic Acid Peanuts

J.C. BRADDOCK, C.A. SIMS and S.F. O'KEEFE

ABSTRACT

A new peanut line has been developed at the University of Florida with about 80% oleic and 3% linoleic acid. Volatiles and sensory characteristics of roasted normal and high oleic acid peanuts stored at 25°C were compared. Volatiles were analyzed using adsorbent trapping and GC-MS, a 20-member trained panel was used for sensory evaluation, and a GC sniffer port was used to evaluate odor characteristics of volatile isolates. Peroxide values were lower for high oleic (HO) peanuts than normal peanuts during storage at 25°C and 40°C. The hexanal content of the peanuts was higher for normal than HO. Peanutty flavor was more stable for HO than normal after 6 wk storage. Painty and cardboard flavors were higher in normal peanuts than HO during storage. Differences for both painty and cardboard flavors were significant after 6 wk storage. Pyrazines were more stable in HO peanuts. Shelf life was estimated from sensory data to be two times longer in HO peanuts.

Key Words: peanuts, oleic acid, flavor stability, pyrazines, peroxide

INTRODUCTION

OXIDIZED FLAVORS generated during storage are important to overall flavor and aroma of roasted peanuts. Final flavor and aroma quality are strongly influenced by oil stability, since most peanut genotypes contain around 50% oil. Peanut oil is composed of ≈ 80% unsaturated fatty acids, with oleic (18:1ω9) comprising an average of 50% and linoleic (18:2ω6) 30% of the total fatty acid composition (Mercer et al., 1990). Because of the polyunsaturated fatty acids, peanuts are susceptible to lipid oxidation. Researchers have described a peanut line with 80% oleic acid and 2–3% linoleic acid, which had improved oil oxidative stability (Moore and Knauft, 1989; O'Keefe et al., 1993).

Many investigators have evaluated the volatile composition of peanuts and identified hundreds of compounds, which may be responsible for the unique roasted flavor. Walradt et al. (1971) identified heterocyclic compounds, sulfur compounds, phenols, ketones, esters, alcohols and hydrocarbons among roasted peanut volatiles. Crippen et al. (1992) evaluated roasted peanut flavor quality by correlating volatiles with gas chromatography and a descriptive sensory panel. That investigation showed pyrazines, methylbutanal, methylpropanal and sulfur compounds (e.g. methanethiol, carbon disulfide and dimethylsulfide) were related to dark roasted flavors. In another study, instrumental and olfactory GC analyses agreed well for good quality (Peroxide Value-PV = 1.4 meq/kg) and oxidized (PV = 111 meq/kg) roasted peanuts (Vercellotti et al., 1992). Bett and Boylston (1992) isolated volatile flavor compounds from roasted peanuts that were stored at 37°C. They reported a mixture of many heterocyclic and other volatile compounds contributed to the characteristic peanut flavor, and storage caused a significant decrease in many heterocyclic compounds responsible for nutty, browned and roasted impressions. They suggested the decline was due to degradation by lipid radicals.

Extending flavor stability during storage would benefit processors and consumers of peanut products, and decreasing losses from lipid degradation should lead to value-added peanut products. Our objective was to identify and compare volatiles and sensory characteristics of roasted normal and high oleic (HO) acid peanuts during storage. Identification of undesirable flavors, measurement of flavor changes and relating sensory descriptors to specific compounds could also help provide an index of overall flavor quality and shelf stability for HO peanuts.

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MATERIALS & METHODS

MATURE PEANUTS from the 1993 crop (high oleic 501/1250 Sunrunner, normal oleic 612/612 Florunner) were obtained from the Univ. of Florida Institute of Food & Agricultural Sciences. Peanuts were shelled and sized on an Official Grading Screen (16/64 in. × 3/4 in). Splits and discolored peanuts were discarded. Peanuts were stored at about 2°C in glass jars under N_2 atmosphere until roasting. The peanuts were roasted (medium roast to Hunter L = 50) with periodic stirring on aluminum baking pans in a conventional oven at 177°C for 50–55 min. Uniform degree of roast was verified with a Hunter Lab Difference/Color Meter (model D25-2).

Storage

Freshly roasted peanuts were placed on aluminum pans and immediately stored in ovens at 25°C (40% relative humidity). For peroxide values, some peanuts were also stored at 40°C (25% relative humidity). The relative humidity was monitored using a Gemware motorized fan psychrometer (model Electro-V, Kahl Scientific Instrument Corp., El Cajon, CA). Samples for testing were removed after storage of 1, 7, 14, 21, 27, 45, 59 and 74 days. Testing was discontinued after sensory panelists detected strong oxidized flavors in samples.

Peanut composition

Moisture contents in raw and roasted nuts were determined according to an official method 27.005 (AOAC, 1984). Lipids were extracted from skinned ground peanuts using methylene chloride according to the method described by O'Keefe et al. (1993). Fatty acid composition of peanut oil was determined by gas chromatography of the fatty acid methyl esters (Maxwell and Marmer, 1983). A 50 m × 0.25 mm i.d., 0.25 μm film BXP-70 (equivalent to 70% cyanopropyl siloxane) fused silica capillary column (Scientific Glass Engineering, Ringwood Australia) was used. Analyses were performed on a Shimadzu GC-14A (Kyoto, Japan) with a flame ionization detector (FID). Samples were injected in triplicate using an initial temperature of 185°C for 20 min. The temperature increased at a rate of 5°C min until a final temperature of 250°C was attained and held at final temperature for 15 min. Linear velocity of helium carrier gas was 22 cm/sec. The split ratio was 40:1. Fatty acid methyl ester standards GLC 68A (20 mg/mL) were used as references (Nu-Chek-Prep, Inc., Elysian, MN).

Sucrose content was measured using a resorcinol method (Roe, 1934). The extract was prepared using ≈ 75 to 100 mg defatted peanut meal. The meal was boiled with 25 to 35 mL 80% ethanol for 1 hr. Resorcinol was obtained from Sigma Chemical Co. (St. Louis, MO).

Extraction of headspace volatiles

A method modified from that described by Vercellotti et al. (1988) was used for volatile extraction. Headspace volatiles were trapped on PrepSep C-18 cartridges (Fisher Scientific, Fair Lawn, NJ). Skinned peanuts were ground with a Waring Blendor for 1 min (mixing every 20 sec) and 200g were placed in a 250 mL extraction flask. The C-18 cartridges were fixed with a rubber stopper to the side-arm of the extraction flask containing the ground peanuts. A vacuum (50 cm Hg)

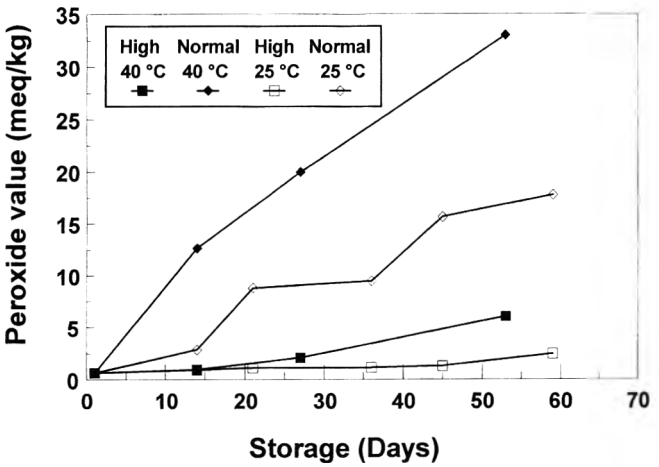

Fig. 1—Peroxide values of roasted peanuts stored at 25°C and 40°C.

applied for 2 hr to the outlet and a small stream of nitrogen gas (≈ 70g/cm²) to the flask inlet allowed vacuum stripping and adsorption of volatiles. Temperature was held at 55°C using a constant temperature water bath. Compounds were eluted from the C-18 column using 500 µL methanol (Fisher, Optima grade) and collected in a 1.0 mL micro reaction vessel using a 12-port extraction vacuum manifold (Supelco, Bellefonte, PA). Extractions were performed in duplicate.

Analysis of volatiles

A gas chromatograph (Perkin-Elmer Sigma 3B, Norwalk, CN) equipped with an olfactory detector outlet (Scientific Glass Engineering, Ringwood, Australia) and an Alltech Econo-cap SE-54 (95% dimethyl-5% diphenylpolysiloxane) capillary column (30m × 0.54 mm i.d., 3 µm film) with helium carrier gas (linear flow velocity 45 cm/sec) was used for analysis of volatiles. Initial column temperature was held at 60°C for 5 min and programmed at 5°C/min to 185°C with a 5 min hold at final temperature. The split ratio was 6:1. An HP 3393A integrator (Hewlett Packard Co., Avondale, PA) was used with attenuation and threshold set to 2. The injector was 225°C and FID 250°C. The column effluent was split 1:1 using a glass Y to FID and olfactory port.

Standard curves were prepared for those compounds identified and determined in preliminary experiments to be important in peanut flavor. Solutions of 2,5-dimethylpyrazine, 2-methylpyrazine, ethyl-pyrazine, 2,3-dihydrobenzofuran, nonylaldehyde, valeraldehyde and phenylacetaldehyde (Aldrich Chemical Co., Milwaukee, WI) were prepared at 10 to 1000 ppm in methanol and injected in triplicate. Linear regression was performed for each standard. Concentrations of other pyrazines for which standards were unavailable were calculated using the regression equation for 2,5-dimethylpyrazine, since comparable responses were observed for pyrazine standards. Peanut volatiles were injected in duplicate into the GC (2.0 µL) and evaluated at various concentrations using the Aroma Extract Dilution Analysis (AEDA) described by Grosch (1990).

GC-MS analysis

Peanut volatiles were concentrated from ≈ 500 µL to 50 µL using a slight stream of N_2 gas at room temperature. One µL of the concentrated aroma was injected into a Hewlett Packard 5890A GC attached to a 5971A MSD Mass Spectrometer. A DB-5 (95% dimethyl-5% diphenylpolysiloxane) capillary column (60 m × 0.25 mm i.d., 0.25 µm film) obtained from J & W Scientific (Folsom, CA) was used. Helium carrier gas was used at 0.64 mL/min and the injector was operated at a split ratio of 5:1. GC-MS parameters were identical to GC conditions for volatile analyses. The spectral analyses were performed with ChemStation computer software program containing the Wiley library (Hewlett Packard Software, Atlanta, GA).

Chemical measurement of oxidation

The iodometric titration procedure was used to measure the peroxide values (AOCS, 1989).

Sensory evaluation panel selection and training

Twenty panel members from the Food Science and Human Nutrition Department, University of Florida were trained with the *Lexicon of Peanut Flavor Descriptors* according to Johnsen et al. (1987). Peanuts with 3 degrees of roast (light, medium and dark) and three stages of oxidation (slight, moderate and extreme) were presented to panelists along with a control (medium roast, not oxidized). Roasted peanutty, raw/beany, cardboardy, painty, sweet, dark roast/toasted and crunchiness were chosen to be the most significant attributes in stored peanuts during initial screening sessions. These descriptors were rated on a 15-point scale at every panel session. Three subsequent sessions were used to train panelists. Trained judges were provided a reference (fresh, medium roasted) and ranges for each attribute. Anchor points for different attributes for control peanuts were as follows: roasted peanutty 9-10, raw/beany 0-1, cardboardy 0-1, painty 0-1, sweet 4-5, dark roast 0-1, crunchiness 10-11. Reference peanuts were roasted, then stored frozen (−20°C) in glass jars in the dark under a N_2 atmosphere. They were brought to 25°C before opening jars. Two coded cups each containing ≈20 g whole nuts of HO and normal peanuts were randomly presented in duplicate. Saltine crackers and deionized water were also provided. Panelists were instructed to remove skins and consume 3–4 nuts at once. Evaluations were conducted in partitioned booths for peanuts removed from storage at days 1, 7, 14, 21, 45, 59 and 74.

Statistical design and analysis

Descriptive sensory evaluation data for the stored peanuts were analyzed using SAS for Windows Version 6.08 (SAS Institute, Cary, NC). A split-plot experimental design with the peanut line designated as the whole plot and storage time as the subplot was used. The panelists were treated as blocks. The day, line and line*day effects were analyzed using ANOVA ($p<0.05$). Means for each time period were compared using Student's t-test.

RESULTS & DISCUSSION

HIGH OLEIC AND NORMAL PEANUTS initially had similar moisture contents, percent sucrose, fat contents and roast colors ($p>0.05$, data not shown). Fatty acid composition of normal and HO samples were similar, except for the varying levels of oleic and linoleic acid. The oleic acid (18:1ω9) content was 79.3% in HO and 55.0% in normal peanuts. Linoleic acid was 2.9% in HO and 24.9% for the normal line. Total saturated fatty acids was 15.4% of HO and 18.4% of normal peanut fatty acids, primarily because of a slightly lower palmitic acid in the HO line.

Peroxide values for the normal samples increased at a faster rate than HO peanuts at both 25°C and 40°C (Fig. 1). Since oxidized flavors were noted by panelists at the third week of storage (25°C) for the normal samples, peroxide values from ≈ 8 to 10 may be useful as an endpoint of flavor quality for roasted peanuts. Therefore, shelf life was estimated as the time to reach the arbitrary peroxide value of 10 from the linear regression of the peroxide value-time curves. Results indicated that HO peanuts had shelf lives of 360 at 25°C and 94 days at 40°C, whereas normal peanuts had shorter shelf lives of 32 and 13 days, respectively. Thus the shelf life was from 11.3 (25°C) to 7.2 (40°C) times longer for HO. We assumed that the PV increase would be linear for the HO peanuts. The AOM induction times for HO and normal peanut oils have been reported to be 9.5 times longer for HO than normal (O'Keefe et al., 1993).

Volatile compounds identified in freshly roasted peanuts were initially present at similar levels in both types of peanuts (data not shown). Aroma responses determined at the GC sniffer port were used to characterize each compound (Table 1). Methylpyrazine provided a savory, grilled chicken odor and 2,5-dimethyl-pyrazine was described as an intense chocolate, malty aroma. Ethylpyrazine was distinctly rich, dark toasted/roasted. A smooth roasted peanutty note was associated with 3-methylpyridine. Other compounds eliciting miscellaneous aromas important to the overall flavor were also isolated. Acetic acid smelled dough-like and benzeneacetaldehyde smelled flowery and sweet. An intense rubbery, harsh, sulfur-like response re-

Table 1—Volatile compounds identified in freshly roasted high and normal oleic acid peanuts

Compound	Sensory Response
1 Acetic acid	bread dough, yeasty
2 Methylpyrazine	grilled chicken, savory
3 2,5-Dimethylpyrazine	malty, chocolate
4 Ethylpyrazine	toasted, dark roasted
5 2,3-Dimethylpyrazine	roasted
6 Benzaldehyde	—
7 2-Ethyl-5-methylpyrazine	nutty, roasted
8 2-Ethyl-3-methylpyrazine	roasted
9 Benzeneacetaldehyde	floral, sweet, caramel
10 3-Ethyl-2,5-dimethylpyrazine	roasted, slightly sweet
11 2,3-Dihydrobenzofuran	rubbery, harsh
12 Benzothiazole	harsh, rubbery, burnt
13 3-Methylpyridine	intense, peanut butter, roasted

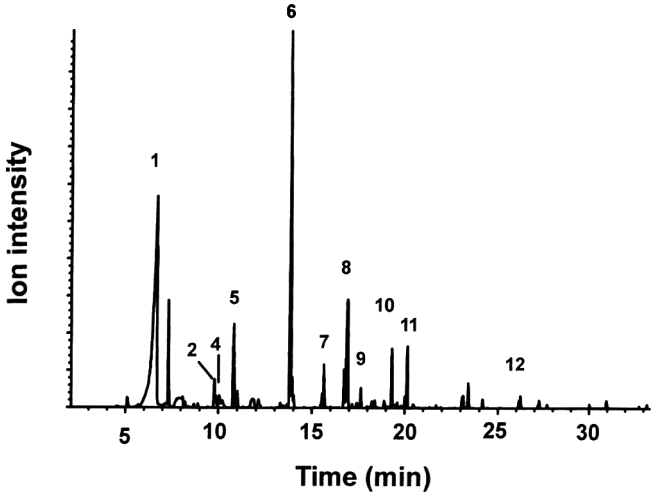

Fig. 2—GC-MS chromatogram of high oleic peanut volatiles after 74 days (25°C). (For peaks identification see Table 1.)

sulted from 2,3-dihydrobenzofuran. Benzothiazole provided a slightly burnt, rubbery off-aroma.

In freshly roasted samples, pyrazines were present in abundant amounts and lipid oxidation products were absent, or only present in trace amounts. A GC chromatogram of volatiles from highly oxidized HO (Fig. 2) and normal (Fig. 3) peanuts (after 74 days at 25°C) were compared. Acetic acid increased with storage time and 2-heptanol was present only in HO samples and after 3 wk storage. After 8 wk, 1-pentanol was found in the normal but not in the HO peanuts. Hexanal was associated with an intense green, grassy off-aroma while nonanal had a powerful floral note at the sniffer port. Differences in aromas of aldehydes generated from oxidation of oleic and linoleic acids could affect the character of the off flavors observed in the two peanut lines. Heptan-2-ol and 1-pentanol contributed much milder odor notes and were characterized by slightly pungent, green aromas.

Aroma thresholds in air reported by Grosch (1990) and the calculated flavor dilution values for volatiles identified in the peanut extracts were compared (Table 2). Compounds determined to be most potent based on higher-FD values, were hexanal, methylpyrazine, 2,5-dimethylpyrazine and nonanal. After 74 days storage, the FD-value for hexanal was 7.3 for HO and 14.3 for normal peanuts. Results suggest that off-flavors due to hexanal were more notable in normal peanuts. Nonanal had a higher FD-value in the HO (5.3) compared to normal peanuts (2.7). This was most likely due to differences in oleic acid contents. Although the extent of oxidation, as measured by peroxide value, was higher in normal peanuts throughout storage (Fig. 1), the lower oleic acid levels in the normal peanuts apparently resulted in generation of lower nonanal levels.

FD-values for pyrazines in the fresh HO samples ranged from ≈1.5 to 8.9 and were lower in those peanuts after 74 days storage. Normal samples tended to have lower pyrazine FD values than HO, ranging from 1.8 to 4.5. Sensory panels detected higher apparent sweetness in HO peanuts after storage, although they had similar sucrose levels. Benzene acetaldehyde did not appear to contribute to increased sweet response in the HO peanuts since its level was higher in normal peanuts. It had an FD-value of ≈ 1.8 at the final storage evaluation in the normal peanut volatiles and an FD-value of < 1 in the HO flavor extracts.

Pyrazines and aldehydes were important in the aroma of roasted peanuts and appear to be key compounds in flavor stability (Ho et al., 1983; Lee et al., 1981; Heath and Reineccius, 1986). Pyrazines such as methylpyrazine, 2,5-dimethylpyrazine, 2,3-dimethylpyrazine and 2-ethyl-5-methylpyrazine were pres-

Table 2—Flavor dilution values of compounds in stored roasted peanuts

Compound	Aroma Threshold in air (ppm)	FD-Value High oleic Day 1	High oleic Day 74	Normal oleic Day 1	Normal oleic Day 74
Hexanal	10	—	7.28	—	14.3
Methylpyrazine	50	4.36	3.37	2.95	2.64
2,5-Dimethylpyrazine	50	8.85	5.92	6.18	4.53
Ethylpyrazine	50	2.46	<1.0	1.52	<1.0
2-Ethyl-5-methylpyrazine	50	3.21	2.66	2.1	1.89
Benzeneacetaldehyde	10	<1.0	<1.0	<1.0	1.8
Nonanal	10	—	5.34	—	2.71

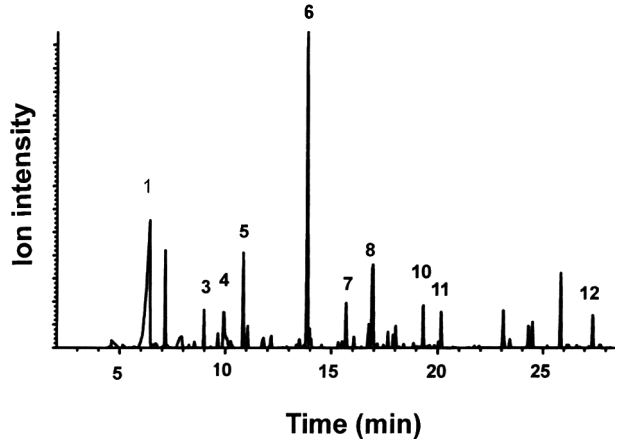

Fig. 3—GC-MS chromatogram of normal oleic peanut volatiles after 74 days (25°C) (For peaks identification see Table 1.)

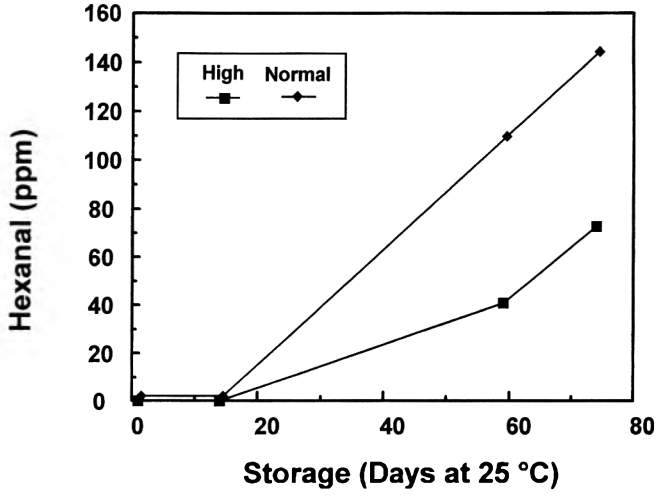

Fig. 4—Change in hexanal concentration in roasted peanuts during storage (25°C).

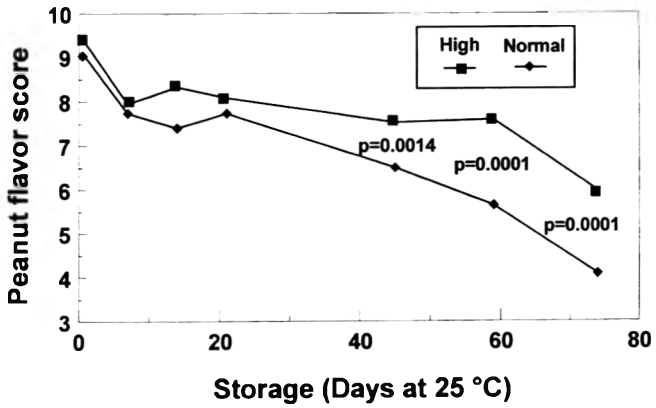

Fig. 5—Change in peanuty flavor score during storage (25°C).

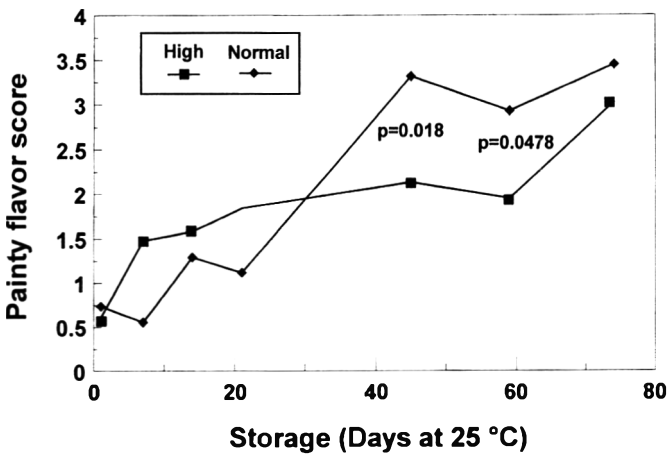

Fig. 6—Change in painty flavor scores during storage (25°C).

ent in higher levels in the HO volatiles after extended storage (data not shown). The change in hexanal concentration with storage (Fig. 4) reflected the lower oxidation in HO peanuts. Hexanal was present in the normal headspace volatiles after 2 wk and was detected at 3 wk in the HO extracts. After 74 days storage, hexanal was 142 ppm in normal and 73 ppm in HO samples. Nonanal, which contributed a mild floral aroma, was detected after the second week in both lines and was determined to be 52 ppm for HO and 27 for normal peanuts at day 74. Since the origin of nonanal is oleic acid, we expected that its level would be higher in the HO peanuts.

Some volatile compounds were fairly stable during storage. For example, benzothiazole and 3-methylpyridine were identified in each storage period and added to overall roasted flavor impact. Some undesirable flavors were not identified but were apparent during sniffer port analyses. Fatty, buttery, stale notes were more pronounced in the oxidized normal peanut samples. Other compounds contributing peanutty, roasted aromas were detected in the HO volatiles but were too low for dependable identification.

Peanut flavors for the two lines were not different for freshly roasted samples. Peanut flavor intensity of the two peanut lines became highly significant ($p<0.01$) after 45 days storage (Fig. 5). The average peanut flavor intensities of stored samples after 74 days storage were 6.0 for HO and 4.1 for normal samples. Since peanut flavor intensity should correlate with sensory quality it may be possible to use this to estimate shelf life. Linear regression calculation of the time to reach an arbitrary end point of 6.0 indicated that the HO had a shelf life of 89 days while for normal peanuts it was 47 days. Thus, estimates suggest that the HO peanut shelf life was almost twice as long as normal peanuts. This was less than the relative difference estimated by

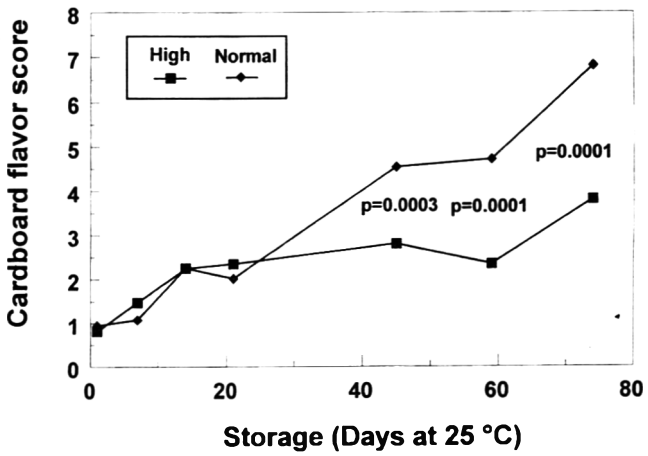

Fig. 7—Change in cardboardy flavor scores during storage (25°C).

peroxide value. Since peroxides are tasteless, their levels may not be an accurate estimator of shelf life.

The dark roasted flavor notes decreased with storage for both lines, and were different only at week 2 ($p=0.021$, data not shown). The overall dark roasted/toasted intensity was < 2 in the freshly roasted samples for both lines. Thus, heterocyclic flavors such as pyrazine and pyridine compounds may not have been formed initially in high enough concentrations to create a very high dark roasted response. Ethylpyrazine appeared to elicit an intense dark roast response at the sniffer port. At the final storage time, almost no dark roasted character was apparent in the two peanut lines. This could be due to a decrease in concentration (below threshold) of overall level of total heterocyclic compounds responsible for dark toasted response, due to destruction by free radicals.

The initial raw/beany flavor was perceived to have the same intensity for both lines and gradually increased to 2.0 for HO and 3.0 for normal peanuts. A difference was observed at the final panel session ($p=0.0026$). The increase could be explained by the loss of roasted flavor components, resulting in apparent intensity of this mild (almost bland) flavor. Certain lipid oxidation flavor compounds such as hexanal have been associated with green peanuts, so an increase in such products could also have generated a slight raw, beany character.

The change in painty (Fig. 6) and cardboardy (Fig. 7) flavors during storage were also compared. A difference was apparent at day 45 of storage for both. The normal sample was higher in cardboardy and painty off-flavors compared to the HO peanuts. No one particular flavor compound was identified at the sniffer port as a specific cardboardy flavor. However, several fatty, stale aromas were observed. Probably the unknown compounds were aldehydes or hydrocarbons.

Crunchiness is an important texture attribute of roasted peanuts and the degree of fatty acid saturation affects melting point of oils. Due to increased oleic to linoleic acid ratio, texture could possibly be affected by differences in fatty acid composition. Crunchiness, determined by sensory evaluation, tended to decrease at the same rate in both lines. No significant difference was detected until the final storage time ($p=0.02$), where the HO samples were slightly more crunchy than normal peanuts (data not shown). Both samples were described as becoming more chewy during storage.

CONCLUSIONS

HIGH OLEIC PEANUTS maintained a more desirable flavor quality during storage due to a slower decline in roasted flavor and less off-flavor development than normal peanuts. Sensory evaluation of peanut flavor and oxidation-derived off flavors paralleled changes in volatile composition (pyrazines and aldehydes) in

stored peanuts. Estimates of shelf life based on sensory evaluation of peanut flavor suggest that roasted HO peanuts would have a shelf life about two times longer than normal peanuts.

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Polyphenoloxidase from Amasya Apple

M. OKTAY, I. KÜFREVIOĞLU, I. KOCAÇALIŞKAN and H. ŞAKIROĞLU

ABSTRACT

Polyphenoloxidase (PPO) of Amasya apple was partially purified by $(NH_4)_2SO_4$ precipitation and dialysis. The sample was used for characterization of the PPO. Optimum pH were 7.0, 9.0, 8.6 and 6.6 on substrates catechol, 4-methyl catechol, pyrogallol and L-dopa respectively. Catechol was the most suitable for Amasya apple PPO. The optimum temperature for maximum PPO activity was 18°C with catechol. Of seven inhibitors tested, the strongest was L-cysteine. Effectiveness of inhibitors increased in the order: thiourea, glutathione, β-mercaptoethanol, sodium cyanide, ascorbic acid, sodium metabisulfide, and L-cysteine. The K_M was 34 mM of catechol. The activation energy with catechol was 107 cal/mol. In electrophoretic separation, three isoenzymes were detected with both catechol and L-dopa substrates.

Key Words: Amasya apple, polyphenoloxidase, isozymes, physical stability

INTRODUCTION

ENZYMATIC BROWNING catalyzed by PPO occurs when plant tissues are damaged, and is an economic problem for processors and consumers. The main step in enzymatic browning is the oxidation of phenolic compounds to corresponding quinones by PPO in the presence of oxygen. The quinones then condense to form darkened pigments (Matheis, 1983).

PPO is a copper-containing enzyme widely distributed in plants (Mayer and Harel, 1979; Mayer, 1987). It has been related to enzymatic browning in several plant tissues, including potato tubers (Patil and Zucker, 1965; Matheis and Belitz, 1978; Walter and Purcell, 1980; Kocaçalişkan and Özbay, 1987) peaches (Jen and Kahler; 1974; Flurkey and Jen, 1980), bananas (Kahn, 1985), grapes (Cash et al., 1976; Valero et al., 1988; Lamikanra et al., 1992), pears (Halim and Montgomery, 1978; Wissemann and Montgomery, 1985), green olives (Ben-Shalom et al., 1977), kiwis (Park and Luh, 1985), strawberries (Ebeling and Montgomery, 1990), plums (Siddiq et al., 1992) and apples (Janovitz-Klapp et al., 1977; Keles, 1986; Coseteng and Lee, 1987). Little has been reported about the Amasya apple PPO. We observed that Amasya apple has one of the highest rates of enzymatic browning among several apple cultivars. PPO from different plant tissues shows different substrate specificities and degrees of inhibition (Vamos-Vigyazo, 1981). Therefore, characterization of the enzyme could help to develop more effective methods in controlling browning of Amasya apples and products. Our objective was to isolate PPO from Amasya apple and study characteristics of the enzyme at different pH and temperatures. Substrate and inhibitor effects were also studied.

MATERIALS & METHODS

Isolation of PPO

Amasya apples (*Malus sylvestris* Miller cv. Amasya) were harvested fresh (in Tortum, Turkey) and stored at 4°C. For preparing the crude extract, 100g of apple tissue was cut quickly into thin slices and homogenized with 200 mL of chilled 0.1M phosphate buffer (pH 7) containing 10 mM ascorbic acid and 0.5% PVP in a Waring Blender for 2 min. The homogenate was filtered and the filtrate was centrifuged at 20,000 × *g* for 30 min at 5°C. The supernatant was brought to 80% $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$. The precipitated PPO was separated by centrifugation at 20,000 × g for 30 min. The precipitate was dissolved in a small amount of homogenization buffer and dialyzed at 4°C in the same buffer for 3 hr with three changes of buffer during dialysis. The dialyzed sample was used as the PPO enzyme source in the following experiments.

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Assay of PPO activity

PPO activity was determined by measuring the increase in absorbance at 420 nm with a spectrophotometer (LKB-Biochrom). The sample cuvette contained 2.9 mL of substrate in various concentrations prepared in the homogenization buffer, and 0.1 mL of the enzyme. The blank sample contained only 3 mL of substrate solution. The reaction was carried out at four temperatures (18, 56, 58 and 70°C), and pH values of 7.0, 9.0, 8.6 and 6.6 using catechol, 4-methyl catechol, pyrogallol and L-dopa substrates, respectively. Enzyme activity was calculated from the linear portion of the curve (Wong et al., 1971). One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001/min. PPO activity was assayed in triplicate measurements. For determining substrate specificity, commercial grades of the substrates were used. L-dopa,catechol,4-methyl catechol and pyrogallol each were added to the standard reaction mixture to give a final concentration of 0.05M.

Effect of pH and temperature

PPO activity was determined using four different substrates (L-dopa, catechol, 4-methyl catechol and pyrogallol) at 0.01M using appropriate buffers (0.1M citrate/0.2M phosphate, 0.1M phosphate and 0.1M Tris-HCl) between pH 3.5 and 10. The determined optimum pH was used in all other experiments. PPO activity was measured at temperatures ranging from 15°C to 80°C by using different substrates. Substrate solution was heated to the tested temperature and the enzyme was added.

Enzyme kinetics

Michaelis constant (K_M) and maximum velocity (V_{max}) were determined using four substrates (L-dopa, catechol, 4-methylcatechol and pyrogallol) in varying concentrations (0.002, 0.004, 0.006, 0.008, and 0.01M) and in optimum conditions (pH, temperature and ionic strength). The reaction was followed in a spectrophotometer and data were plotted according to Lineweaver and Burk (1934).

Effect of inhibitors

To determine effects of inhibitors, PPO activity was measured using seven different inhibitors at three concentrations with catechol substrate (Table 3). Results were expressed as Ki constants and percent catechol inhibition.

Activation energy

Enzyme activity log values from temperature assays for each substrate were plotted as related to the reciprocal of absolute temperature. The activation energies of reactions were calculated from the slopes. The Arrhenius equation was used for determining activation energies.

Electrophoresis

Polyacrylamide gel slab electrophoresis was performed according to the method of Laemmli (1970) to separate PPO isoenzymes of Amasya apple. The electrode buffer was tris/glycine (pH 8.3) using 3% stacking gel and 10% seperating gel. The enzyme sample was loaded (0.08 mL, 0.044 mg protein) into each space of the stacking gel. Initially, an elec-

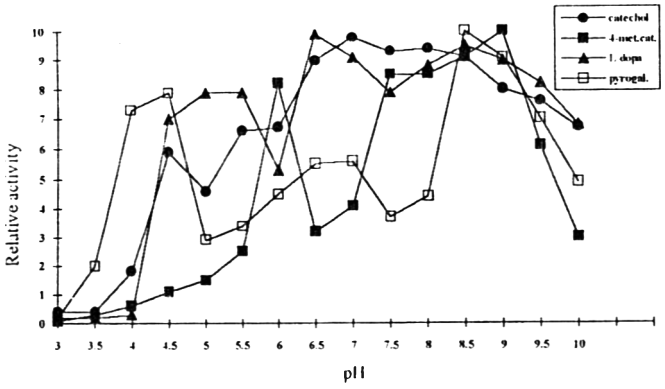

Fig. 1—Effect of pH on PPO activity.

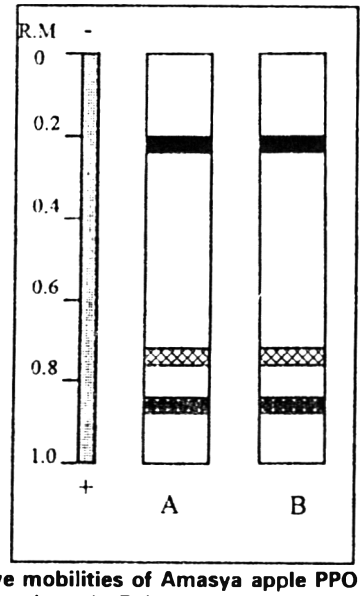

Fig. 2—Relative mobilities of Amasya apple PPO on polyacrylamide gel electrophoresis. Relative mobility was based on migration of bromophenol marker dye. Isoenzyme bands were developed using catechol (A), and L-dopa (B) substrates. Color intensities of the bands: ■ strong, ▪ moderate, ▫ weak (R.M: Relative mobility).

Table 1—Substrate specificity of Amasya apple PPO

Substrate (0.05 mM)	Activity (units/mL)
Catechol	1187.0
4-Methyl catechol	520.2
L-dopa	24.2
Pyrogallol	781.0

tric voltage (80 V) was applied until the bromphenol dye reached the seperating gel. Then it was increased to 200 V for 3 hr. Electrophoresis was carried out at 4°C. After completion of the run, the gel was cut into two symmetrical parts. One part was immersed in 0.1M phosphate buffer (pH 6) containing 1.5 mM L-dopa substrate, and the other part immersed in phosphate buffer (pH 7.2) containing 15 mM catechol and 0.05% o-phenylenediamine. The isoenzyme bands were developed for 1 hr and each gel then rinsed in 1 mM ascorbic acid solution for 5 min. The gels treated with L-dopa and catechol substrates were stored in distilled water or 30% ethanol respectively, and photographed.

RESULTS & DISCUSSION

Effect of pH

Different pH optima were determined for each substrate (Fig. 1). Maximum PPO activities were at pH 7.0 for catechol, 9.0 for 4-methyl catechol, 8.6 for pyrogallol, and 6.6 for L-dopa. In general, most fruit showed maximum activity at or near neutral pH values (Betrosian et al., 1960; Chan and Yong, 1971; Wong et al., 1971; Cash et al., 1976; Siddiq et al., 1992). In our study, we found similar results for catechol and L-dopa substrates but pH optima of the other two substrates were alkaline. We found three peaks in the pH-activity graphs. This may indicate different isoenzymes, since we found three isoenzymes of Amasya apple PPO (Fig. 2).

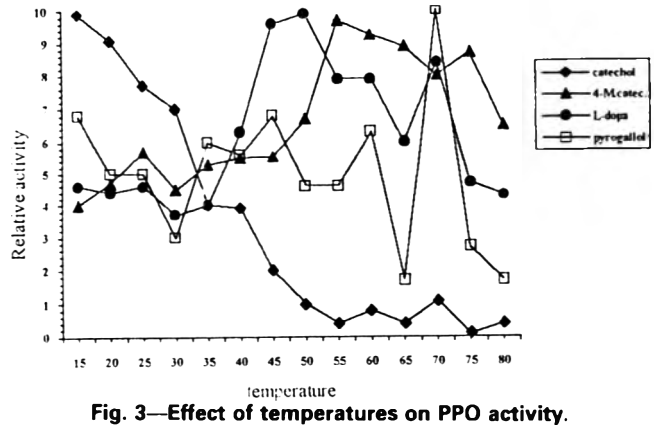

Fig. 3—Effect of temperatures on PPO activity.

Table 2—K_M and V_{max} values for each substrate

Substrate	K_M (mM)	V_{max} (units/mL)
Catechol	34.0	2000
4-Methyl catechol	3.1	552
L-dopa	6.5	27
Pyrogallol	27.0	1215

Substrate specificity

Amasya apple PPO showed activity with all the o-diphenolic substrates (Table 1). The substrate with highest activity was catechol, followed by 4-methyl catechol, pyrogallol and L-dopa. In earlier studies, however, the most suitable substrate for PPO was reported to be 4-methyl catechol and the least effective was pyrogallol in apple chloroplasts (Harel et al., 1965) and peel (Stelzig et al., 1972).

Effect of temperature

Optimum temperatures for maximum PPO activity (Fig. 3) were 15°C for catechol, 56°C for 4-methyl catechol, 50°C for L-dopa, and 70°C for pyrogallol. When catechol was substrate, PPO showed maximum activity at 15°C and decreased gradually with increasing temperatures. Above 60°C PPO showed almost no activity. With other substrates, however, PPO showed fluctuations in activity with increasing temperatures even as high as 80°C.

Enzyme kinetics

Values calculated from Lineweaver-Burk graphs (Table 2) showed K_M values were 34 mM for catechol, 3.1 mM for 4-methyl catechol, 6.5 mM for L-dopa, and 27 mM for pyrogallol. From these results, we suggest Amasya apple PPO has more affinity for 4-methyl catechol than for the other substrates. In an earlier work (Walker, 1964), K_M of apple chloroplast PPO was reported to be 1.6 mM with chlorogenic acid substrate. However, K_M of apple flesh PPO has been indicated as 4.6 mM with catechol substrate (Vamos-Vigyazo, 1981).

Effect of inhibitors

Percent inhibition and Ki values for the inhibitors we used (Table 3) indicated that thiourea and glutathione show uncompetetive inhibition whereas the other inhibitors were compete-

Table 3—K_i constants and percent inhibition values of the PPO with different inhibitors

Inhibitor	Concentrations (M)	K_i (M)	% Inhibition
β-mercaptoethanol	1.38×10^{-3}	1.8×10^{-2}	4.0
	5.50×10^{-3}	1.5×10^{-2}	22.3
	8.30×10^{-3}	1.1×10^{-2}	36.8
Thiourea	3.3×10^{-5}	7.8×10^{-4}	2.5
	9.9×10^{-5}	6.5×10^{-4}	4.4
	1.32×10^{-4}	4.6×10^{-4}	6.3
Sodium cyanide	8.9×10^{-5}	1.4×10^{-4}	33.1
	1.8×10^{-4}	1.2×10^{-4}	53.1
	2.6×10^{-4}	1.1×10^{-4}	65.6
Sodium metabisulphide	1.7×10^{-5}	9.6×10^{-6}	15.8
	3.3×10^{-5}	8.1×10^{-6}	76.7
	4.9×10^{-5}	6.7×10^{-6}	85.4
Glutathion	2.04×10^{-5}	4.0×10^{-4}	1.3
	4.08×10^{-5}	2.1×10^{-4}	3.5
	8.17×10^{-5}	1.1×10^{-4}	12.2
L-cysteine	9.7×10^{-6}	9.8×10^{-6}	42.5
	2.9×10^{-5}	6.5×10^{-6}	77.6
	4.8×10^{-5}	4.2×10^{-6}	90.0
Ascorbic acid	1.7×10^{-5}	9.2×10^{-5}	12.5
	3.5×10^{-5}	7.3×10^{-5}	26.9
	7.1×10^{-5}	5.1×10^{-5}	74.1

Table 4—Activation energies (E_A) of PPO for different substrates

Substrate	Slope ($-E_A/2.303R$)	Activation energy (cal/mol)
Catechol	0.235	−107.3
4-Methyl catechol	−0.062	28.3
Pyrogallol	0.044	−20.0
L-dopa	−0.046	20.9

tive. The most effective inhibitor was L-cysteine. In a reported study (Janovitz-Klap et al., 1990), cysteine was a strong inhibitor of apple PPO. Since L-cysteine is a naturally occurring amino acid and nontoxic, it may be useful in preventing enzymatic browning of apple products.

Activation energy

For each substrate, a plot was constructed by using temperature-activity values from the temperature optimum study. Activation energy (E_A) was calculated from the slopes (Table 4). Activation energies of catechol and pyrogallol were negative whereas all others were positive. This may be explained by inactivation of the PPO by high temperature. Activation energy values are, generally, not published, therefore comparisons are not possible. Only one study of PPO in kiwifruit states its activation energy was 7 kcal/mol with catechin substrate (Park and Luh, 1985).

Electrophoresis

Three isoenzymes were separated by polyacrylamide slab gel electrophoresis and detected using catechol and L-dopa substrates (Fig. 3). In both cases, the slow moving first bands were more active than the fast moving second and third bands (indicated by color intensity). Previous studies indicate three PPO isoenzymes were found in apples (Harel et al., 1965; Constantinides and Bedford, 1967).

CONCLUSIONS

PPO OF AMASYA APPLE showed different pH optima with different substrates. Its thermostability was quite high, even as high as 80°C. The most effective inhibitor of PPO activity was L-cysteine. Since L-cysteine is a naturally occurring amino acid and nontoxic, it may be of use in preventing enzymatic browning of apple products. Three PPO isoenzymes were detected by electrophoresis.

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Enzymatic Browning of Model Solutions and Apple Phenolic Extracts by Apple Polyphenoloxidase

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ABSTRACT

Model solutions containing chlorogenic acid (CG) and (−)-epicatechin (EP), and phenolics from nine apple cv., were oxidized with apple polyphenoloxidase (PPO). Browning was determined by absorbance at 380–700 nm, and transformed into CIE L*, a* and b*. Multilinear correlations were established between initial and degraded amounts of each phenolic. In model solutions of CG and EP, color values and A_{400} correlated strongly with initial and oxidized CG and EP. Both phenolics contributed to decrease in L* and increase in A_{400}. Increase in CG increased a*, but EP had little effect. The impact of increased EP on b* was two to three times higher than for CG. In each phenol class (hydroxycinnamic (HD), flavan-3-ol (FA), flavonol (FO) and dihydrochalcone (DC) derivatives), degraded amounts were increased or decreased by increasing amounts of other classes. Besides major phenolics, HD (mainly CG) and FA (mainly EP and procyanidin B2), FO and DC, appeared to affect color development.

Key Words: polyphenoloxidase, chlorogenic acid, epicatechin, apple phenolics, browning

INTRODUCTION

ENZYMATIC BROWNING occurs in fruits and vegetables after bruising, cutting or during storage. This results from oxidation of phenolic compounds by polyphenoloxidase (PPO), in the presence of oxygen, into quinones (Macheix et al., 1990). The *o*-quinones of various phenolic compounds have great differences in stability and underwent subsequent reactions (further oxidation and polymerization) leading to dark colored pigments (Richard-Forget et al., 1992; Rouet-Mayer et al., 1993). With few exceptions, this reaction is undesirable due to development of unpleasant colors and flavors and loss of nutrients. Researchers have attempted to correlate browning intensity with main parameters responsible, i.e., phenolic content and enzymatic (PPO) activity (Macheix et al., 1990). Studies on different apple cultivars have shown contradictory results (Nicolas et al., 1994). The susceptibility to browning may depend on PPO activity (Vamos-Vigyazo et al., 1976; Coseteng and Lee, 1987), phenolic content (Harel et al., 1966; Macheix, 1970; Coseteng and Lee, 1987), or both (Prabha and Patwardhan, 1985) but with variable relative influence during fruit growth (Macheix, 1970) on any parameters (Klein, 1987).

Conflicting results illustrate complex interactions among the parameters involved in apple enzymatic browning and limits of methods used to evaluate it (Nicolas et al., 1993). The relative proportions of different classes of phenolic substrates in apple are important for the final color of oxidized tissues (Nicolas et al., 1994). Apple cortex contains four classes of phenolics. Hydroxycinnamic (HD) and flavan-3-ol (FA) derivatives have been identified as the most important (Amiot et al., 1992). Among them, chlorogenic acid (CG), (−)-epicatechin (EP) and procyanidin B2 (PcB2) were prominent. Other classes such as flavonol glycosides (FO) and dihydrochalcone derivatives (DC) have been reported in lower amounts (Amiot et al., 1992). Apple PPO had a different specificity towards these substrates. CG, EP and (+)-catechin were considered as good substrates (Richard-Forget, 1992). Janovitz-Klapp et al. (1990a) showed that apple PPO was at least twice more active with CG than with EP or (+)-catechin. Other phenolics could be considered as poor substrates or inhibitors. Apple PPO slowly degraded procyanidin B2 (Goodenough and Lea, 1979). In apple, the degree of browning is dependent on the balance of HD and FA, and on PPO activity (Amiot et al., 1992). Among HD, CG is the best natural substrate of apple PPO (Janovitz-Klapp et al., 1990a) and gives *o*-quinones which can cooxidize many other substances (Richard-Forget, 1992). FA are also substrates of PPO and yield pigments which are more intensely colored than those from CG (Rouet-Mayer et al., 1990). Moreover, their degradation rates are enhanced by CG, mainly by coupled oxidation reactions (Oszmianski and Lee, 1990; Richard-Forget, 1992). PPO activity and other minor phenolics together with ascorbic acid content and acidity may also influence browning (Amiot et al., 1992).

Two approaches have been proposed to evaluate degree of browning. Measurements based on absorbance spectrophotometry or tristimulus colorimetry have been applied on cut surfaces, purées or juices (Aubert et al., 1992). Some workers have used the decrease in luminance (ΔL^*) to measure degree of browning (Burda et al., 1990). Others have used changes in L* and a* (Sapers et al., 1990), in L*, a* and b* (Mastrocola et al., 1989), or calculated a color vector difference ΔE ($\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$) (Rhim et al., 1989). However, neither ΔL^* nor ΔE give precise representation of resulting colors. Values were very dependent on methods and optical conditions of measurement, and on physical state of examined samples. Other methods used the absorbance measurement of soluble pigments by spectrophotometry at 360–500 nm, often near 400 nm (Rouet-Mayer et al., 1990; Goupy et al., 1991). Absorbance measurements only slightly correlated with visual evaluation of browning since oxidized, insoluble polymerized pigments bound to cell wall membranes were omitted. Browning products, resulting from PPO oxidation of phenolics, vary widely in color intensity and hue, and depend on type of phenolics (Rouet-Mayer et al., 1990). The susceptibility of apple to browning could be adequately determined by simultaneous measurements of soluble (absorbance at 400 nm or A_{400}) and insoluble (lightness or L*) brown pigments (Amiot et al., 1992). Soluble pigments correlated with HD degraded whereas insoluble pigments correlated with FA degraded.

Use of chromometers have become extensive and the need to relate L*, a* and b* values to enzymatic browning is clear. Our objectives were to develop oxidation model systems and correlate absorbance and CIE Lab parameters with initial and oxidized phenolic amounts.

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MATERIALS & METHODS

NINE APPLE CULTIVARS were grown in the experimental orchard of the Centre Technique Interprofessionnel des Fruits et Légumes of Balandran near Avignon (France) (CA: Reinette du Canada; EL : Elstar; FL : Florina; FU : Fuji; GA : Gala; GO : Golden; GS : Granny Smith; JO : Jonagold; RD : Red Delicious). Apples were picked at commercial

Table 1—Equations relating tristimulus color parameters L*, a* and b* to initial (in) and oxidized (ox) chlorogenic acid (CG) or (−)-epicatechin (EP) in single phenolic solutions obtained by linear regression analysis

	Initial amount		Oxidized amount	
	CG_{in}	EP_{in}	CG_{ox}	EP_{ox}
A_{400}	$0.03 + 0.53\ CG_{in}$ r = 0.95	$0.01 + 2.03\ EP_{in}$ r = 0.99	$0.03 + 6.6\ CG_{ox}$ r = 0.97	$-0.01 + 2.9\ EP_{ox}$ r = 0.99
L*	$99.2 - 15.4\ CG_{in}$ r = 0.99	$99.3 - 26.9\ EP_{in}$ r = 0.97	$99.4 - 18.8\ CG_{ox}$ r = 0.99	$99.6 - 38.9\ EP_{ox}$ r = 0.98
a*	$-0.83 + 12.7\ CG_{in}$ r = 0.97	$-3.17 - 5.4\ EP_{in}$ r = 0.92	$-0.97 + 15.4\ CG_{ox}$ r = 0.97	$-3.09 - 7.9\ EP_{ox}$ r = 0.94
b*	$2.10 + 34.4\ CG_{in}$ r = 0.99	$5.6 + 81.7\ EP_{in}$ r = 0.99	$1.65 + 42.2\ CG_{ox}$ r = 0.99	$4.68 + 118\ EP_{ox}$ r = 0.99

Table 2—Equations relating tristimulus color parameters L*, a* and b* to initial (in) and oxidized (ox) chlorogenic acid (CG) and (−)-epicatechin (EP) in binary mixtures obtained by multilinear regression analysis

	Initial amount CG_{in} and EP_{in}	Oxidized amount CG_{ox} and EP_{ox}
L*	$99 - 21.0\ CG_{in} - 25.2\ EP_{in}$ r = 0.96	$98.5 - 19.6\ CG_{ox} - 30.4\ EP_{ox}$ r = 0.97
a*	$-4.21 + 27.9\ CG_{in} + 0.48\ EP_{in}$ r = 0.95	$-4.57 + 31.8\ CG_{ox} + 2.0\ EP_{ox}$ r = 0.95
b*	$2.58 + 51.5\ CG_{in} + 98.2\ EP_{in}$ r = 0.98	$4.62 + 41.3\ CG_{ox} + 118.3\ EP_{ox}$ r = 0.99

maturity and stored at 4°C for 2 mo. Fruits were peeled and seeds were removed. The flesh was frozen in liquid nitrogen, and crushed. Powders were stored at −20°C until required.

CG and EP were of reagent grade from Sigma and 4-methylcatechol from Fluka. All other chemicals were of reagent grade supplied by Merck.

Multilinear regression analysis were carried out using a program developed according to Poole and Borchers (1979).

Extraction and purification of apple PPO and apple phenolics

The PPO was purified 120-fold from the cortex of RD according to the method used by Janovitz-Klapp et al. (1989). PPO activity was measured by polarography with a Clark electrode using 4-methylcatechol (20 mM) as substrate in air saturated solutions at 30°C. Activity was expressed in nanomoles of oxygen consumed/sec (nanokatals or nkat). Extraction and purification of apple phenolic compounds were carried out according to Amiot et al. (1992), with minor modifications. Metabisulfite was omitted in ethanol solution (65%) since this compound is a powerful inhibitor of apple PPO (Janovitz-Klapp et al., 1990b). Simultaneous measurements of oxygen uptake, colored products and phenolic oxidations were carried out. All assays were performed in duplicate. Results are means of the 2 values which did not differ by more than 5%.

Oxidation by apple PPO

Prior to oxidation, each methanolic extract was evaporated under vacuum and the residue dissolved in a McIlvaine buffer solution at pH 4.5. Model solutions (5 mL) of CG and EP at concentrations ranging from 0 to 0.5 mM by 0.1 mM step, were prepared in McIlvaine buffer at pH 4.5 containing 0.2 mM vanillic acid as internal standard. The maximum concentration of 0.5 mM for CG and EP was chosen since their concentrations were usually below this limit in apple cortex (Nicolas et al., 1994). Oxidation was carried out at 30°C for 30 min with 25 nkat of purified apple PPO in a total volume of 5 mL in the presence of air bubbling in the solution.

The enzymatic reaction was fixed at 30 min with 25 nkat of PPO in 5 mL of solution; since after this period, the spectrum between 380 and 700 nm (and consequently the L*, a* and b* values) remained constant. During oxidation, solutions were continuously aerated.

Assay of phenolic compounds before and after enzymatic oxidation

After 30 min oxidation, the enzymatic reaction was stopped by addition of an equal volume of solution containing 2 mM NaF (Richard-Forget et al., 1992) in 80% water adjusted to pH 2.6 with *o*-phosphoric acid (solvent A) and 20% acetonitrile (solvent B) to model solution of CG and EP, or 20% of a mixture of acetonitrile and methanol; (1:1.5, v:v) (solvent C) to apple phenolics. The phenolic compounds before and after 30 min oxidation were separated and quantified by HPLC (9010 pump and 9050 UV detector driven by a 9020 workstation from Varian) on 10 µL samples using an analytical column (Lichrospher RP18 endcapped, 125 mm, 4.0 mm i.d., 4 µm particle size from Merck). For model solutions, isocratic conditions (90% A and 10% B) were used at 1 $mL \cdot min^{-1}$. CG and EP were assayed at 280 nm using vanillic acid as internal standard.

For the apple cv experiment, the best separation was obtained at 33°C (flow rate 1 $mL \cdot min^{-1}$) by the following gradient: 0–20 min, 10% C; 20–40 min, 32% C; 40–50 min, 32% C; 50–55 min, 40% C; 55–60 min, 50% C. Phenolic compounds were assayed by external standard calculation using CG for hydroxycinnamic derivatives (HD), EP for flavan-3-ol derivatives (FA), quercetin for flavonol derivatives (FO) and phloridzin for dihydrochalcone derivatives (DC).

RESULTS & DISCUSSION

Oxidation of CG and EP by apple polyphenoloxidase

Amounts of oxidized CG (CG_{ox}) and EP (EP_{ox}) were determined in each tested solution and correlated with their initial contents (CG_{in} and EP_{in}) by multilinear regression:

$$CG_{ox} = 0.01 + 0.86*CG_{in} - 0.04*EP_{in} \quad r=0.99 \quad (A)$$

$$EP_{ox} = 0.01 + 0.14*CG_{in} + 0.84*EP_{in} \quad r=0.99 \quad (B)$$

with $0 \leq CG_{in} \leq 0.5$ mM and $0 \leq EP_{in} \leq 0.5$ mM.

The amount of CG_{ox} was mainly related to CG_{in}, but slightly decreased as EP_{in} was increased (Equation A); whereas, the amount of EP_{ox} increased with increasing amounts of EP_{in} and CG_{in}, six times faster with EP_{in} than with CG_{in}. This protective effect of EP towards oxidation of CG and the acceleration of EP degradation were probably due to the fact that *o*-quinones of CG could oxidize EP through a non enzymatic coupled reaction leading to regeneration of CG and further oxidation of EP (Oszmianski and Lee, 1990; Richard-Forget et al., 1992).

Relationships between initial and degraded phenols and tristimulus data

Model solution with single phenolic. Correlations with A_{400} were slightly better with EP than with CG content (Table 1). An increase in phenolic content led to a decrease of L* values and an increase of A_{400} values. Variations in L* and A_{400} were higher with EP_{in} and EP_{ox} than with CG_{in} and CG_{ox} (between two and four times for the same initial concentration). Thus for pure phenolic solution, EP gave more intense coloration than CG at the same concentration.

Oxidized CG gave higher a* solutions whereas oxidized EP gave lower a* solutions. This is illustrated in the a* equations by the positive factor for CG_{in} and CG_{ox}, and the negative factor for EP_{in} and EP_{ox} (Table 1). These results confirmed those obtained by Rouet-Mayer et al. (1990) and Richard-Forget et al. (1992). They found that CG gave *o*-quinones which were dull orange-yellow with maximum absorbance at 420 nmn with secondary oxidation products that were lighter. After oxidation, o-quinones from EP (or CA) were bright yellow with maximum absorbance at 380 nm. These *o*-quinones were much less stable and colored than those formed from CG at the first phase of the reaction. In the second phase of the reaction the secondary products were darker than the *o*-quinones.

Table 3—Amounts of phenolic compounds in each class, initial (in) and oxidized (ox) before and after PPO oxidation for nine apple cultivars[a]

	HD[b]		FO[b]		FA[b]		DC[b]	
Cultivar	in	ox	in	ox	in	ox	in	ox
Reinette Canada (CA)	0.495	0.188	0.046	0.040	0.412	0.162	0.032	0.019
Elstar (EL)	0.139	0.070	0.014	0.002	0.312	0.192	0.045	0.030
Fiorina (FL)	0.112	0.062	0.010	0.007	0.115	0.053	0.049	0.031
Fuji (FU)	0.271	0.179	0.040	0.010	0.357	0.187	0.070	0.059
Gala (GA)	0.423	0.149	0.030	0.022	0.466	0.180	0.071	0.030
Golden (GO)	0.261	0.149	0.019	0.007	0.255	0.089	0.060	0.047
Granny Smith (GS)	0.119	0.026	0.021	0.011	0.424	0.115	0.053	0.045
Jonagold (JO)	0.215	0.092	0.028	0.025	0.246	0.068	0.056	0.042
Red Delicious (RD)	0.340	0.226	0.038	0.023	0.458	0.252	0.065	0.054

[a] Values are given in concentration (mM) of initial and degraded phenols in the solution for each cultivar. (Mean values are duplicated experiments).
[b] HD = hydroxycinnamic, FA = flavan-3-ol, FO = flavonol, DC = dihydrochalcone derivatives.

Table 4—Tristimulus parameters of oxidized solutions of phenolic extracts from nine apple cultivars[a]

Cultivar	L*	a*	b*
Reinette Canada (CA)	90.8	2.64	34.3
Elstar (EL)	96.1	−0.80	15.7
Florina (FL)	97.3	−0.48	10.8
Fuji (FU)	91.9	0.03	36.4
Gala (GA)	92.8	0.32	29.9
Golden (GO)	91.7	1.02	33.4
Granny Smith (GS)	98.1	−0.77	8.4
Jonagold (JO)	94.9	−0.29	18.5
Red Delicious (RD)	89.4	0.96	49.7

[a] Mean values are duplicated experiments.

Model solutions with two phenolics. L*, a* and b* values were related in binary mixtures to amounts of CG and EP (initial or oxidized) by multilinear regression (Table 2). Correlation coefficients were very similar for both initial and oxidized amounts of CG and EP. These results obviously indicated strong correlations between these two parameters (see equations A and B). The factors affecting CG_{in} and CG_{ox} on the one hand, and EP_{in} and EP_{ox} on the other hand, are of the same order of magnitude. However, the influence of EP_{ox} on L*, a* and b* variations was always higher than that of EP_{in} which again could be related to the acceleration in EP oxidation caused by *o*-CG-quinones.

For equal amounts of phenols (both initial and oxidized), the impact on L* variations was always higher for EP than for CG (Table 2). However, in both, the presence of a second phenolic increased the negative coefficient associated with the first phenolics (e.g. − 15.4 in Table 1 and −21 in Table 2 for CG_{in}). This increase was larger for CG than for EP. Thus, in binary mixtures, the impact of EP on L* variations was only 25% (initial amount) and 50% (oxidized amount) higher than CG (Table 2) compared to the 200% observed in single phenolic solutions (Table 1).

Similar effects were observed on b* variations for initial amounts of phenolics since the presence of the second phenolic increased the positive factor associated with the first phenolic. The impact of EP on a* values compared to CG was almost nil (Table 2).

Thus, in CG and EP mixtures, both phenolics contributed to L* and b* variations. The observed effect was always larger compared to that in single phenolic solutions. For a* variations, the CG impact was more than doubled whereas that of EP was almost nullified. Therefore, both phenolics contributed to darkening and yellowness with a higher impact of EP than of CG, whereas CG was mainly responsible for redness in oxidized binary solutions. The first result confirmed observations of Oleszek et al. (1989) reporting that CG seemed less significant in color development than FA. In our model systems and experimental oxidation conditions, insoluble pigments were not present and therefore did not affect absorbance measurements.

Oxidation of phenolic extracts by purified apple polyphenoloxidase

For each cv, the phenolic contents of the solution were determined before and after enzymatic oxidation to obtain the initial and degraded amounts of phenolic compounds, respectively (Table 3). Concerning initial amounts, results were in agreement with those reported by others (See Nicolas et al. (1994) for a review of phenolic composition of apple cortex). Mainly four classes of phenolics were present, HD, FA, FO and DC. HD and FA were main phenolics in the cortex of the 9 cv since they represented between 80 and 90% of the total phenolics. CG was the major compound in HD with exception of Florina where cryptochlorogenic acid (4'-caffeoyl quinic acid) was very high (data not shown). EP and PcB2 were the most important in the FA (data not shown) in agreement with the results of Burda et al. (1990). In the two other classes, FO (quercetin derivatives) and DC (mainly phloridzin) were present only in small quantities.

In agreement with Amiot et al. (1992), the four classes of phenolics were affected by enzymatic oxidation (Table 3). CG and EP were the best natural phenolic substrates for apple PPO (Janovitz-Klapp et al., 1990a) whereas other apple phenolics were not substrates or were ineffective (Richard-Forget, 1992). The degradation of apple phenolics could be the result of coupled oxidation by *o*-quinones enzymically formed mainly from CG (Nicolas et al., 1994). Such coupled oxidations have been reported in binary model solutions containing a caffeoyl derivative (caftaric acid or CG) and a flavan-3-ol (Cheynier et al., 1989 and 1991; Oszmianski and Lee, 1990; Richard-Forget, 1992) or flavonol (Richard-Forget, 1992) or a dihydrochalcone (Oszmianski and Lee, 1991; Richard-Forget, 1992).

Relationships between amounts of initial phenols and degraded phenols. In order to compare the effects of each class of phenols on degradation of other classes, the amounts of initial and oxidized phenolics within each class were normalized. We set the highest value for each class equal to 1 and the lowest equal to zero. For HD_{in}, the normalized values were as follows: CA = 1, EL = 0.07, FL = 0, FU = 0.415, GA = 0.812, GO = 0.389, GS = 0.018, JO = 0.269 and RD = 0.595. After normalization, correlations between amounts of oxidized phenolics (HD_{ox}, FA_{ox}, FO_{ox} and DC_{ox}) and initial amounts (HD_{in}, FA_{in}, FO_{in} and DC_{in}) were sought by multilinear regression analysis as follows:

$$HD_{ox} = 0.05 + 0.55HD_{in} + 0.45FO_{in} - 0.25FA_{in} + 0.29DC_{in} \quad r = 0.89$$
$$FO_{ox} = 0.22 + 0.43HD_{in} + 0.42FO_{in} - 0.05FA_{in} - 0.30DC_{in} \quad r = 0.88$$
$$FA_{ox} = -0.08 - 0.05HD_{in} + 0.15FO_{in} + 0.69FA_{in} + 0.08DC_{in} \quad r = 0.78$$
$$DC_{ox} = 0.09 - 0.87HD_{in} + 0.84FO_{in} - 0.07FA_{in} + 0.68DC_{in} \quad r = 0.91$$

FA_{ox} amounts were always poorly predicted ($r < 0.80$) even when the four classes were taken into account.

Conversely, HD_{ox} amounts seemed adequately predicted as soon as HD_{in} was considered, the validity increasing when the number of classes increased, resulting in a strong correlation ($r = 0.89$) when all classes were taken into account. For the FO_{ox} amounts, only when at least two classes (FO_{in}/HD_{in} or FO_{in}/DC_{in}) were considered were correlation coefficients > 0.83. Similarly for the DC_{ox} amounts, three classes were needed (DC_{in}, HD_{in} and FO_{in}) to obtain a strong correlation ($r = 0.91$).

When the impact of each class of initial phenolic was considered, amounts of oxidized phenolics in one class positively

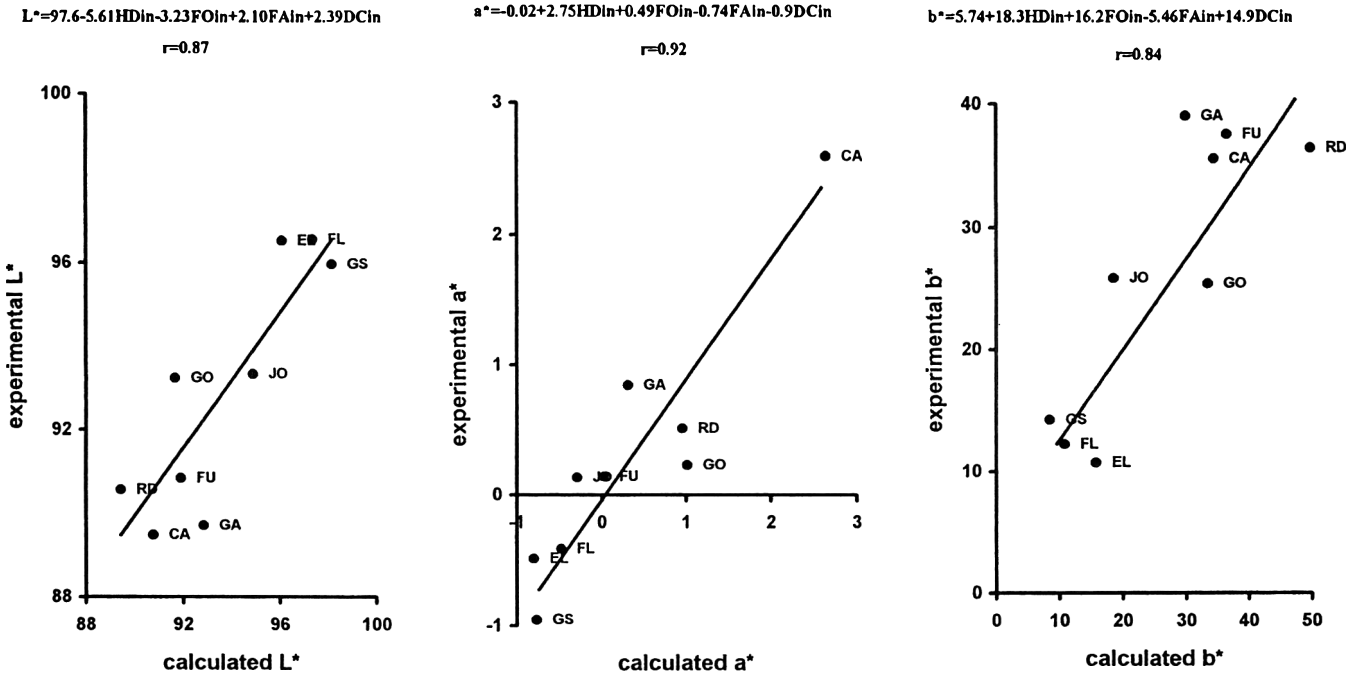

Fig. 1—Comparison of experimental and calculated values of L*, a* and b* using the equations determined with initial amounts of HD, FO, FA and DC contents. Apple cultivars CA = Reinette Canada, EL = Elstar, FL = Florina, FU = Fuji, GA = Gala, GO = Golden, GS = Granny Smith, JO = Jonagold, RD = Red Delicious.

correlated with the initial phenolics of the same class. For HD_{ox} amounts, FO_{in} and DC_{in} exhibited a positive contribution to HD_{ox}, whereas FA_{in} had a negative contribution. This could be due to the coupled oxidation of EP (present in FA) by *o*-quinones of CG leading to a protection of CG against oxidation by EP in binary CG/EP mixtures. When the 4 classes were considered, the effects of HD_{in} and FO_{in} were close and higher than those of DC_{in} and FA_{in}.

For FO_{ox}, the contributions of FO_{in} and HD_{in} were positive and almost equal, whereas that of DC_{in} was negative and that of FA_{in} almost nil. Coupled oxidations of flavonol derivatives by *o*-quinones of CG have been demonstrated (Richard-Forget, 1992), which could explain the positive impact of HD_{in} on FO_{ox}. The protective effect of dihydrochalcone derivatives on flavonol derivatives have never been noted. For DC_{ox}, an important impact of both HD_{in} and FO_{in} (higher than that of DC_{in}) was observed. However, FO_{in} increased the amount of DC_{ox} and HD_{in} decreased it. This was different from findings of Oszmianski and Lee (1991), who indicated that addition of CG accelerated the reaction of phloretin glucoside oxidation.

Color parameters of oxidized phenolic extracts by purified polyphenoloxidase. The tristimulus data, L* (lightness), a* (greenness/redness) and b* (blueness/yellowness), measured on the nine apple phenolic extracts after 30 min oxidation by purified apple PPO were compared (Table 4). For the apple cultivars, the L* values decreased from 98 to 90 and the b* values ranged from 50 to 8.4 whereas small variations were observed for a* values which ranged from 2.64 to −0.80. Similar results have been obtained with binary mixtures of CG and EP after enzymatic oxidation. Moreover, note that the cultivars which had a positive value for a* (namely RD, FU, GO, GA and CA) exhibited the lowest values for L* (<93) and the highest values for b* (≥30). These cultivars were also characterized by the highest A_{400} (data not shown). That observation was also made by Amiot et al. (1992) who indicated that among 11 apple cv those with low lightness frequently gave a high value for the A_{400} and *vice versa*. Lastly, for the nine cultivars, a strong correlation was apparent between L* and b* values (r = −0.98) whereas correlations were lower between L* and a* values (r = −0.80) or the a* and b* values (r = 0.69).

Relationships between amounts of initial phenols and tristimulus data. Correlations obtained by multilinear regression analysis between amounts of initial phenols in the four classes and tristimulus data, L*, a* and b* were compared (Fig. 1). Concerning L*, when only one class of phenols was considered, only HD_{in} had r > 0.80. Moreover, when HD_{in} was not considered, correlations were always weak (FO_{in} : r = 0.76, FA_{in} : r = 0.46, DC_{in} : r = 0.26). When other classes were considered together with HD_{in}, the r value increased and a strong correlation was obtained with the four classes:

$$L^* = 97.6 - 5.61HD_{in} - 3.23FO_{in} + 2.10FA_{in} - 2.39DC_{in} \qquad r = 0.87$$

Three classes (HD_{in}, FO_{in} and DC_{in}) had a negative impact, i.e. decreased luminance of oxidized solution whereas FA_{in} had a positive impact. The highest impact corresponded to HD_{in}. This confirmed that HD derivatives were the most important factor for the decrease in luminance in oxidized apple tissue and confirmed findings of Amiot et al. (1992). An opposite effect was observed for FA_{in}. This was different from the impact of EP found in binary (CG/EP) model solutions since both EP_{in} and CG_{in} decreased L* values. This discrepancy could be due to the fact that a great part of FA_{in} was PcB2 and other oligomers of procyanidins. These phenolics, once oxidized, had a tendency to precipitate and were not considered in the browning measurement.

A similar pattern was found for a* variations. Again, low correlations (r < 0.80) were obtained when HD_{in} was omitted (FO_{in} : r = 0.69, FA_{in} : r = 0.34, DC_{in} : r = 0.27), and strong correlations (r = 0.91) were obtained when both HD_{in} and DC_{in} were considered. When all four classes of phenols were considered, the highest impact was found for HD_{in} followed by DC_{in}, FA_{in} and then FO_{in}:

$$a^* = -0.02 + 2.75HD_{in} + 0.49FO_{in} - 0.74FA_{in} - 0.90DC_{in} \qquad r = 0.92$$

Increasing amounts of HD_{in} gave redder solutions in agreement with findings for model solutions containing CG and EP. Conversely, greener solutions were obtained with increasing amounts of DC_{in} and FA_{in}.

Concerning b* variations, the impacts of all classes of phenols were more important than for L* and a*. Only when at least two classes DC_{in}/HD_{in} or DC_{in}/FO_{in} were considered were the r values > 0.80 (respectively 0.81 and 0.80). When the four clas-

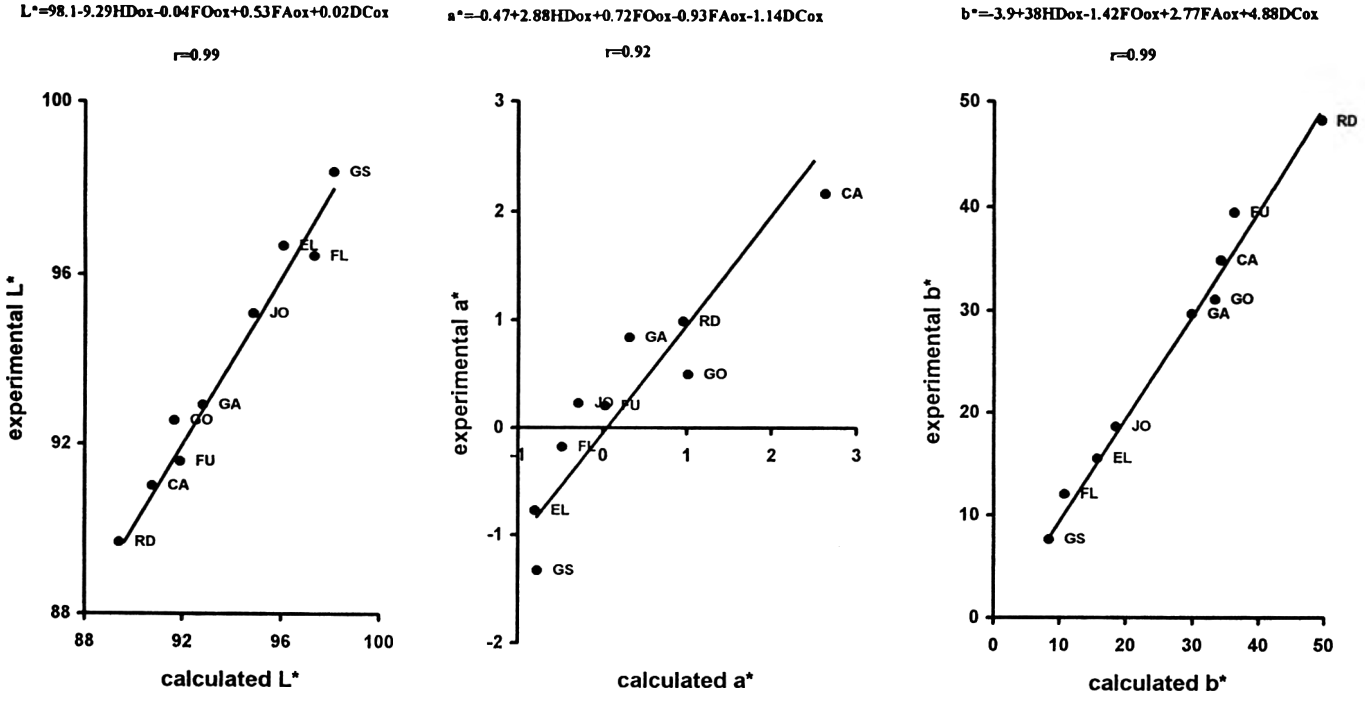

Fig. 2—Comparison of experimental and calculated values of L*, a* and b* using the equations determined with oxidized amounts of HD, FO, FA and DC contents. Apple cultivars CA = Reinette Canada, EL = Elstar, FL = Florina, FU = Fuji, GA = Gala, GO = Golden, GS = Granny Smith, JO = Jonagold, RD = Red Delicious.

ses of phenols were used:

$$b^* = 5.74 + 18.3HD_{in} + 16.2FO_{in} - 5.46FA_{in} + 14.9DC_{in} \qquad r = 0.84$$

HD_{in}, FO_{in} and DC_{in} combined showed a positive impact three times higher than FA_{in} alone, which was negative. Therefore, HD_{in}, DC_{in} and FO_{in} gave yellower solutions in contrast with FA_{in} which slightly decreased b* values. This was different from observations in model solutions (CG/EP), where both increasing amounts of EP_{in} and CG_{in} increased b* values.

Relationships between amounts of degraded phenols and tristimulus data. Correlations by multilinear regression between amounts of degraded phenols in the four classes and tristimulus data, L*, a* and b* were compared (Fig. 2). The L* values correlated strongly to HD_{ox} ($r = 0.99$). If HD_{ox} was not considered, the correlations were always low (FO_{ox}, $r = 0.52$, FA_{ox}, $r = 0.58$, DC_{ox}, $r = 0.22$). The impact of HD_{ox} on L* variations was very large compared to those of other classes.

$$L^* = 98.1 - 9.29HD_{in} - 0.04FO_{in} + 0.53FA_{in} + 0.02DC_{in} \qquad r = 0.99$$

Therefore, it appears that lightness of the 9 oxidized apple phenolic extracts was mainly related to amounts of degraded hydroxycinnamic derivatives. Compared to correlations with HD_{in}, those calculated with HD_{ox} were always stronger.

Opposite results were found with a* values. Thus, correlations with amounts of degraded phenols were always less than or equivalent to those obtained with initial amounts. Moreover, two classes of degraded phenols were needed at least to obtain correlation coefficients higher than 0.80 (HD_{ox}/FO_{ox} : $r = 0.86$, HD_{ox}/FA_{ox}, : $r = 0.81$ and HD_{ox}/DC_{ox} : $r = 0.88$). The impact of HD_{ox} was the highest followed by DC_{ox}, FA_{ox} and FO_{ox} i.e. a similar ranking to that with initial phenolics:

$$a^* = -0.47 + 2.99HD_{ox} + 0.72FO_{ox} - 0.93FA_{ox} - 1.14DC_{ox} \qquad r = 0.92$$

Similarly to initial amounts, HD_{ox} and FO_{ox} gave redder solutions with positive values whereas greener solutions were obtained by increasing amounts of DC_{ox} and FA_{ox} with negative values.

Concerning b*, correlations with HD_{ox} were largely increased ($r = 0.98$) compared to those obtained with HD_{in} ($r = 0.73$). Similarly to results for the L* variations, very strong correlations were obtained ($r \geq 0.98$) as soon as HD_{ox} was included. When the four classes of phenols were considered, HD_{ox} had the highest impact:

$$b^* = 3.92 + 38HD_{ox} - 1.42FO_{ox} + 2.77FA_{ox} + 4.88DC_{ox} \qquad r = 0.99$$

In all cases, the L*, a* and b* values of oxidized solutions were adequately predicted by amounts in phenolics either initial or degraded since r values were always > 0.80. However, predictions of L* and b* values were always greatly improved when amounts of degraded phenolics were considered ($r = 0.99$). Amiot et al. (1992) indicated that the degree of browning of 11 apple cultivars strongly correlated with amounts of HD_{ox} and FA_{ox}.

CONCLUSIONS

DEGREE OF BROWNING OF APPLE PHENOLICS is related to amounts of degraded phenolics. For constant PPO activity, the oxidized amounts could be adequately predicted provided initial amounts were known. Due to coupled oxidation, the oxidized amounts within one class could be increased or decreased by increasing amounts in the other phenolic classes. Therefore, for equivalent total phenolic contents, the resulting pigments can vary both quantitatively and qualitatively leading to widely different values in L*, a* and b*. HD derivatives (mainly CG) had major influence on apple browning. Other phenolic classes influenced variations of L*, a*, and b* of oxidized solutions, by modulating the extent of HD_{ox} and producing different pigments.

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Kinetics of Ascorbic Acid Loss and Nonenzymatic Browning in Orange Juice Serum: Experimental Rate Constants

J.R. JOHNSON, R.J. BRADDOCK, and C.S. CHEN

ABSTRACT

The kinetics of ascorbic acid loss and nonenzymatic browning in clarified orange juice (serum) were investigated in an anaerobic environment from 70.3 to 97.6°C and from 11.7 to 80.6°Brix. Data were fitted to first-order kinetic models. Rate constants of ascorbic acid degradation in serum were not different from rate constants in whole juice. Activation energies were ≈30 kcal/mol and largely independent of solids concentration. Rate constants of browning pigment formation were 30–50% greater in serum. Activation energies were 19–25 kcal/mol and increased slightly with solids concentration.

Key Words: orange juice, nonenzymatic browning, juice serum, ascorbic acid, kinetics

INTRODUCTION

FRUIT JUICE PROCESSING includes the production of juice and essence concentrates as valuable co-products (Chen et al., 1993). The most common evaporator used in the citrus industry for producing orange juice concentrate is the thermally accelerated short time evaporator (TASTE). Prolonged exposure to high temperatures during evaporative concentration promotes degradation reactions in foods resulting in poor quality final products. Two commonly occurring degradations in fruit juices are nonenzymatic browning and ascorbic acid loss.

Nonenzymatic browning in citrus juices is due to the reactions of sugars, amino acids, and ascorbic acid (Nagy et al., 1989). It leads to formation of a wide variety of end products including organic acids, furans, furanones, ketones, cyclopentanones, pyranones, and pyrroles. Many of these compounds contribute to off-flavors in the juice (Nagy et al., 1989).

The explanation for orange juice browning is debatable. The most common method for characterizing browning is the measurement of color development as a function of time and its expression in terms of kinetics of the reactions. Many researchers have hypothesized the appearance of brown pigments as following either zero-order or first-order kinetics (Warmbier et al., 1976; Saguy et al., 1978b; Stamp and Labuza, 1983; Toribio and Lozano, 1986; Buera et al., 1987; Barbanti et al., 1990; Cohen et al., 1994). Others have suggested that assuming the reaction zero- or first-order was simplistic (Petriella et al., 1985; Nagy et al., 1990). Since a variety of browning pigments with varying stabilities are formed (Rouseff et al., 1989), monitoring the color change might be misleading. The statistical difference between a zero-order fit of kinetic data and a first-order fit is very slight (Labuza and Riboh, 1982). Therefore, we hypothesized that either model could be used without introducing significant error.

Ascorbic acid degradation in orange juice contributes to the formation of browning pigments (Handwerk and Coleman, 1988; Lee and Nagy, 1988). Ascorbic acid and dehydroascorbic acid probably enter the browning pigment formation pathway as highly reactive α-dicarbonyls (Handwerk and Coleman, 1988). The degradation is known to occur by both oxidative and nonoxidative mechanisms and is generally characterized as a first-order reaction (Saguy et a ., 1978a; Robertson and Samaniego, 1986). The rate of oxidative degradation has been determined to be from 10 to 1000 times faster than the nonoxidative pathway (Huelin, 1953; Kefford et al., 1958; Braddock and Sadler, 1989).

Most published data on kinetics of orange juice degradation have been reported on conventional whole orange juice. Our objective was to characterize the kinetics of nonenzymatic browning and ascorbic acid degradation of membrane-clarified juice serum at temperatures normally used in conventional evaporation processes.

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MATERIALS & METHODS

Materials

Concentrated serum was prepared using an ultrafiltration membrane system and a pilot-scale TASTE evaporator. The membrane system was manufactured by Koch Membrane Systems, Inc. (Wilmington, MA) and consisted of three polysulfone hollow-fiber membrane cartridges arranged in parallel. Each cartridge had an effective mass transfer area of 4.84 m^2. Individual fibers had an internal diameter of 75 mils. The molecular weight cutoff was 5×10^5. The evaporator was a five-stage, five-effect unit (Cook Machinery, Dunedin, FL) with a water removal capacity of 225 kg/hr.

Sample preparation

Concentrated serum of 80.6°Brix was diluted to make samples of 50.3, 29.9, and 11.7°Brix with deionized water. Whole orange juice concentrate was likewise diluted and used as a control. Known quantities of sample were placed into 9 mL screw cap test tubes (Corning Glass Works, Corning, NY) for heating: 2g of concentrate, 3g of 50.3°Brix, 4g of 29.9°Brix, and 8g of single-strength juice 11.7°Brix. Sample headspaces were purged for 30 sec with nitrogen to create a substantially anaerobic environment and the tubes were capped.

Twelve to 16 tubes of similar samples were prepared for each temperature/concentration combination.

Thermal treatment

Tubes were placed in a constant temperature circulator (Model MR-3220A-1, Blue M Electric Company, Blue Island, IL) for heating. Samples were heated at 97.6, 91.2, 82.0, and 70.3°C. Temperature was monitored with a Digi-Sense thermocouple thermometer (Cole-Parmer Instrument Company, Chicago, IL). Two tubes were removed every 15–30 min and rapidly cooled in an ice water bath. One tube was used for measurement of ascorbic acid content and one was used for measurement of browning index. In this manner, the retention of ascorbic acid and the development of browning pigments could each be monitored as related to heating time. Reaction rates in serum were measured in triplicate, in whole juice twice.

Measurement of browning index and ascorbic acid content

For our study, the browning index for a juice or serum sample was defined as its light absorbance at 420 nm. A Spectronic 21 spectrophotometer (Bausch & Lomb, Rochester, NY) was used for all absorbance measurements. Serum samples were analyzed directly by measuring absorbance. No further clarification was necessary. Concentrated serum samples were diluted to single-strength prior to measurement of absorbance. The absorbance of whole juice samples was measured by the method of Meydev et al. (1977) with slight modification. Whole juice

Table 1—Characteristics of juices used in kinetics studies

Sample[a] #	Conc (°Brix)	% Titratable acidity	pH	% pulp by volume	Viscosity (mPa·s)
1	14.6	1.26	3.6	17	4.41
2	12.4	1.16	3.5	11	4.52

[a] Both samples are early season Valencia. Serum was produced from sample #1. Sample #2 was control.

samples were centrifuged for 10 min at 326 × *g* in a clinical centrifuge to remove sinking pulp. The supernatant was diluted 1:1 with absolute ethanol and allowed to stand 1 hr. An additional centrifugation at 326 × *g* for 10 min completed clarification. Absorbance of the supernatant was measured at 420 nm. Concentrated samples were diluted to 12°Brix prior to analysis.

Ascorbic acid contents were measured by the dichloroindophenol dye method of AOAC as adapted (Redd et al., 1986). The dye was not standardized as absolute ascorbic acid contents were not required. Only relative change in content due to heating or thermal processing was required.

Juice characterization

Soluble solids (°Brix), titratable acidity, pH, % pulp, and viscosity were measured using conventional tests (Redd et al., 1986). All concentrates were diluted to single-strength prior to pH, acidity, and pulp measurements. Single-strength whole juice viscosity was measured at 25°C with a model LVT Brookfield viscometer (Brookfield Engineering Laboratories, Stoughton, MA) utilizing the ultralow viscosity adapter at 30 rpm. Scale measurements were converted to viscosities expressed in units of mPa·s (centipoise).

Model for degradation kinetics

A general reaction rate expression for degradation kinetics can be written as follows (Labuza and Riboh, 1982):

$$-d[D]/dt = k[D]^m \quad (1)$$

where [D] is the quantitative value of quality factor or the undesirable products of the degradation reaction, k is the reaction rate constant, and m is the order of the reaction.

The integral form of a first-order reaction is obtained by integrating equation (1) for m = 1 and can be written as the following expression:

$$\ln([D]_o/[D]_t) = kt \quad (2)$$

where $[D]_o$ is the value of the quality factor at time 0 and $[D]_t$ is the value after reaction time t.

Temperature dependence

The relationship of reaction rate to temperature was quantified by the Arrhenius relationship:

$$k = A_o \exp(-E_a/RT) \quad (3)$$

where E_a is the activation energy of the reaction, R is the gas constant, T is absolute temperature, and A_o is a pre-exponential constant. Solids concentration can be expected to have an effect on parameters of the Arrhenius expression for degradation reactions such as ascorbic acid loss (Saguy et al., 1978a) and nonenzymatic browning (Saguy et al., 1978b). Empirical expressions relating E_a and A_o to solids concentration were developed from experimental data.

RESULTS

Juice characteristics

The physical characteristics of the juices used in the kinetic studies were compared (Table 1). Serum was produced from sample #1 while sample #2 was used as control. Both juices were early season Valencia (note relatively low pH).

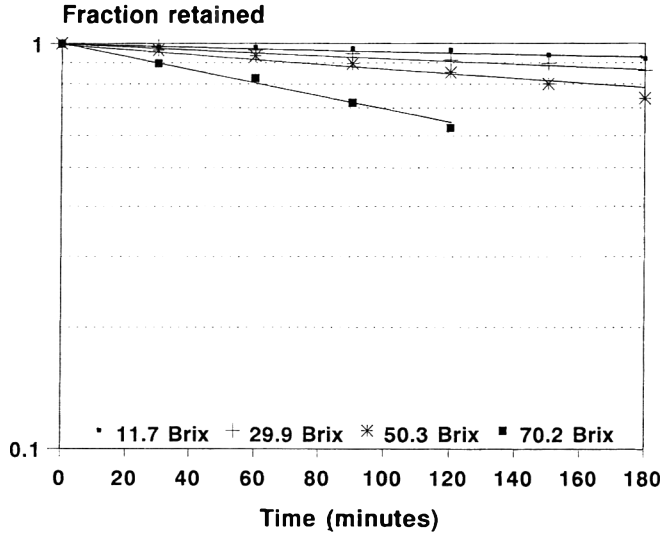

Fig. 1—Changes in ascorbic acid retention in orange juice serum with increased heating time at 91.1°C under anaerobic conditions.

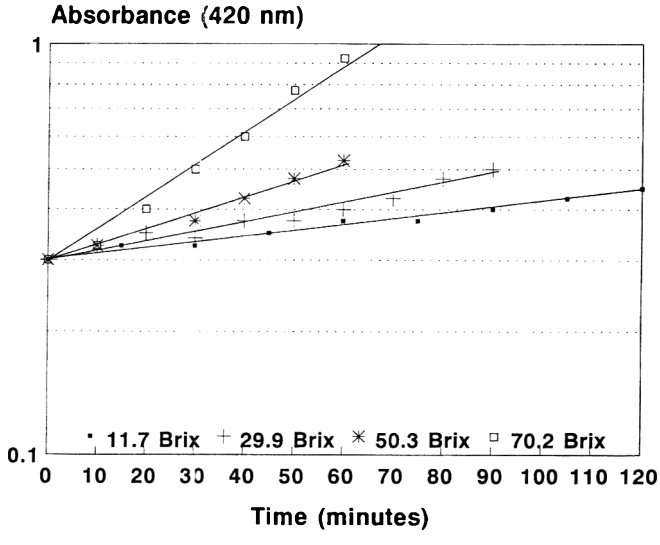

Fig. 2—Changes in absorbance at 420 nm of orange juice serum with increased heating time at 91.1°C under anaerobic conditions.

Ascorbic acid loss

When ascorbic acid concentration was measured as a function of heating, the rate constant could be calculated from Eq. (2) by computer programs. Alternatively, defining [D] in Eq. (2) as ascorbic acid concentration, the rate constant could also be determined from a semi-log plot of $[D]_t$ vs time (see Fig. 1). Linear regression analysis showed r^2 ranging from 0.974 to 0.990. The slope of this straight line plot equals the opposite of the rate constant:

$$\log[D]_t = -(k/2.303)t + \log[D]_o \quad (4)$$

Rate constants for ascorbic acid degradation in orange juice serum and in orange juice (control) as functions of concentration and temperature were calculated by computer and compared (Table 2). Percent losses of vitamin C were 1.2 ± 1.0% for serum and 1.9 ± 1.3% for whole juice. No difference in degradation rate was apparent between whole juice and juice serum. The values of the rate constant were comparable, though lower, to those values reported by Saguy et al. (1978a) for degradation in grapefruit juice. They were also near to values reported by

Table 2—Rate constants for anaerobic ascorbic acid degradation in orange serum and in whole orange juice

	Orange serum		Orange juice	
Temp (K)	Conc (°Brix)	Rate constant $\times 10^{-4}$(1/min)	Conc (°Brix)	Rate constant $\times 10^{-4}$(1/min)
343.5	12.7	0.43	12.5	0.45
343.5	37.3	0.91	36.7	1.45
343.5	55.8	1.16	55.4	1.29
343.5	80.6	2.89	71.6	2.74
355.2	12.7	2.34	12.5	2.99
355.2	37.3	3.31	36.7	4.16
355.2	55.8	6.23	55.4	6.17
355.2	80.6	18.4	71.6	11.2
364.4	12.7	5.47	12.5	8.86
364.4	37.3	9.27	36.7	10.6
364.4	55.8	17.7	55.4	18.2
364.4	80.6	46.9	71.6	34.5
370.8	12.7	10.3	12.5	11.3
370.8	37.3	20.3	36.7	17.4
370.8	55.8	29.6	55.4	26.6
370.8	80.6	80.0	71.6	48.9

Table 3—Activation energies and reaction constants for ascorbic acid degradation in orange serum and in whole orange juice

Product	°Brix	Temp range (°C)	Reaction constant ln A_0	Activation energy (Kcal/g-mol)	r^2
Orange serum	12.7	70.3–97.6	32.8	29.2	0.991
	37.3	70.3–97.6	32.5	28.6	0.998
	55.8	70.3–97.6	35.5	30.3	0.992
	80.6	70.3–97.6	37.2	30.8	0.985
				Avg. 29.7 ± 0.87	
Orange juice	12.5	70.3–97.6	35.2	30.7	0.968
	36.7	70.3–97.6	25.3	23.3	0.998
	55.4	70.3–97.6	33.2	28.7	0.998
	71.6	70.3–97.6	32.2	27.5	0.989
				Avg. 27.6 ± 2.7	

Nagy and Smoot (1977) for the anaerobic degradation of ascorbic acid in canned orange juice.

Assuming an Arrhenius relationship between rate constant and temperature, activation energies, and reaction velocity constants were calculated (Table 3). The activation energies for degradation in juice serum were on the order of 30 kcal/mol and appeared to be independent of solids concentration. This was higher than reported by Nagy and Smoot (1977) or Saguy et al. (1978a). However, it was within the range 15–50 kcal/mol expected for typical chemical reactions (Thijssen and van Oyen, 1977).

Nonenzymatic browning pigment formation

The extent of nonenzymatic browning in serum was quantified by measuring juice absorbance at 420 nm. The specific brown pigments formed were not as much concern as the rate at which they formed. This rate could be correlated with the appearance of a reddish-yellow color. Monitoring at 420 nm is commonly used for determining the distribution of browning pigments in orange juice (Sawamura et al., 1991). Semi-log plots of absorbance vs heating time at 92°C were compared for four concentrations of orange juice serum. Linear regression analysis showed r^2 ranged from 0.950 to 0.985. The linear plots indicated that the absorbance increases could be described as first-order without introducing significant error.

Therefore, the following equation was used to describe the appearance of brown pigments in serum, as measured by absorbance at 420 nm, as a function of heating time:

$$B = B_o \exp(kt) \quad (5)$$

where B = absorbance of the serum at time t; B_o = absorbance of the serum at time 0; k = reaction rate constant in min^{-1} and t = heating time (min).

Table 4—Rate constants for anaerobic nonenzymatic browning in orange serum and in whole orange juice

	Orange serum		Orange juice	
Temp (K)	Conc (°Brix)	Rate constant $\times 10^{-4}$ (min)	Conc (°Brix)	Rate constant $\times 10^{-4}$ (min)
343.5	12.7	10.5	12.5	4.1
343.5	37.3	12.2	36.7	6.7
343.5	55.8	13.8	55.4	10.3
343.5	80.6	24.5	71.6	11.7
355.2	12.7	22.7	12.5	17.0
355.2	37.3	27.9	36.7	19.5
355.2	55.8	39.4	55.4	29.3
355.2	80.6	87.9	71.6	44.1
364.4	12.7	51.6	12.5	34.8
364.4	37.3	79.9	36.7	48.7
364.4	55.8	112.4	55.4	76.0
364.4	80.6	229.8	71.6	123.7
370.8	12.7	75.6	12.5	84.8
370.8	37.3	102.7	36.7	96.0
370.8	55.8	151.4	55.4	136.1
370.8	80.6	347.1	71.6	218.4

Table 5—Activation energies and reaction constants for browning in orange serum and in whole orange juice

Product	°Brix	Temp range (°C)	Reaction constant ln A_0	Activation energy (Kcal/g-mol)	r^2
Orange serum	12.7	70.3–97.6	20.5	18.7	0.994
	37.3	70.3–97.6	23.7	20.7	0.981
	55.8	70.3–97.6	27.2	23.1	0.989
	80.6	70.3–97.6	30.7	25.0	0.995
				Avg. 21.9 ± 2.4	
Orange juice	12.5	70.3–97.6	32.0	27.1	0.991
	36.7	70.3–97.6	28.6	24.5	0.997
	55.4	70.3–97.6	28.3	24.1	0.998
	71.6	70.3–97.6	33.4	27.4	0.999
				Avg. 25.8 ± 1.5	

Rate constants for nonenzymatic browning in orange juice serum, as determined by measuring absorbance at 420 nm, as related to temperature and concentration were compared (Table 4). Values are the averages of three measurements. Variation between measurements averaged <10%. Browning rates for whole orange juice (control) were also compared. Values are averages of two measurements. Variation between measurements averaged 18%. Activation energies and reaction velocity constants were also compared (Table 5). Activation energies were 19–25 kcal/mol, increasing with solids concentration. These values were consistent with those reported for browning in grapefruit juice (Saguy et al., 1978b; Smoot and Nagy, 1980; Cohen et al., 1994).

Our results were different from previous reports at low or common storage temperatures which had suggested nonenzymatic browning proceeded via a zero-order reaction preceded by an initial induction period where little color was formed (Saguy et al., 1978b; Barbanti et al., 1990; Lozano, 1991). An induction time of 2 to 5 min at this temperature has been reported for 62.5°Brix grapefruit concentrate (Saguy et al., 1978b). Some researchers have suggested that describing nonenzymatic browning as strictly zero- or first-order was not valid (Petriella et al., 1985; Nagy et al., 1990). Rather, the reactions are much more complex and the true order may be between zero and one. Possibly nonenzymatic browning reactions more nearly follow first-order kinetics as reactant concentration increases. Absorbance increases at lower serum concentrations had a more linear relationship to heating time.

Rates of browning reactions reportedly decreased in the presence of galacturonic acid (Porretta, 1991). Therefore, browning rates would be expected to be lower in whole juice. However the work of Lozano (1991) with apple juice conflicted with this hypothesis since galacturonic acid increased the rate of browning pigment formation in that study.

CONCLUSIONS

IN ULTRAFILTRATION CLARIFIED orange juice serum, measured quality changes with heat and time were comparable to those

for whole juice. The rate of ascorbic acid degradation in orange serum was almost at the same rate as in whole orange juice. Activation energies for degradation in juice serum were on the order of 30 Kcal/g-mol and largely independent of solids concentration. The rate of browning index increase was greater in juice serum than in whole orange juice. Activation energies were 19–25 Kcal/mol and increased slightly with solids concentration.

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Stability of Non-Pasteurized, Refrigerated Muscadine Grape Juice

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ABSTRACT

The stability of nonpasteurized muscadine grape juice processed with and without 100 mg/L potassium metabisulfite was monitored during storage at 3°C. The muscadine flavor intensity, sweetness, off-flavor levels, and color of white juices remained stable, and ethanol levels remained low for 7 wk. However, the flavor intensity and sweetness of nonsulfited white juice decreased, and off-flavor and ethanol levels increased after 7 wk. Sulfite lowered microbial levels in white juice throughout 9 wk. Red juices did not develop significant levels of off-flavors or ethanol during 9 wk, although the flavor intensity declined, especially in nonsulfited red juice. Sulfite lightened the color of red juice, but the color was stable in both sulfited and nonsulfited red juices.

Key Words: muscadine grapes; red grapes; flavor; storage stability; sulfites

INTRODUCTION

JUICES FROM MUSCADINE GRAPES *(Vitis rotundifolia)* can have excellent and unique flavors (Flora, 1977a; King et al., 1988), and have potential for processors in the Southeast where these grapes are indigenous and commercially cultivated (Olien, 1990). Previous research on muscadine grape juices has shown that heat extraction and pasteurization, as well as high storage temperatures, may reduce the overall quality of muscadine juices (Bates and Gursky, 1979; Flora, 1976a,b; 1977a,b; 1979; Sistrunk and Morris, 1982). The flavor and color of muscadine juices, both red and white, are sensitive to heat and research has indicated that sterile filtration can produce a high quality, stable juice (King et al., 1988). Grape maturity and the sugar:acid ratio can also affect juice quality (Carroll and Marcy, 1982; Carroll et al., 1971; Johnson and Carroll, 1973; Lanier and Morris, 1978).

Interest and commercial development are growing in the production and marketing of high-quality, non-pasteurized, refrigerated juices (Brown et al., 1993). Although shelf-life of some such products (e.g. orange juice) is limited due to microbial growth (Parish, 1988; Teller, 1993), initial flavor is excellent (Fellers, 1988). Such methods of production and marketing may be well suited to muscadine grape juice which is also sensitive to heat, has a relatively low pH (usually below 3.4), and lacks polyphenoloxidase (Sims et al., 1991), which should result in very little browning of fresh juice. However, research is needed on overall flavor and color stability of both red and white muscadine juices, as well as the potential value of sulfite in these products to prevent discoloration and help control microbial growth. Sulfite is typically added to pasteurized white grape juices, but its value in nonpasteurized juices is unknown. Our objectives were to assess the stability of nonheated muscadine juices, processed with and without sulfite, in refrigerated storage.

MATERIALS & METHODS

HAND-HARVESTED RED (cv. Noble) and white (cv. Welder) muscadine grapes were obtained from a local vineyard and crushed in a grape destemmer-crusher within 5 hr of harvest. The crushed grapes were either treated with 100 mg/L sulfite or not treated, and the white grapes were pressed immediately in a small bladder press. The crushed red grapes were treated with a commercial macerating enzyme system (Cytolase 219, 200 mg/kg, Genencor, San Francisco, CA) for 3 hr at 21°C and then pressed. The juices were clarified (slightly) by settling overnight at 3°C, but were not cold stabilized to precipitate tartrates. The juices were bottled into 1.9L plastic milk cartons, capped and stored at 3°C. All treatments were duplicated.

The juices were evaluated initially and every week for 9 wk. The juices were subjected to sensory evaluation by a 10 member panel consisting of students, faculty and staff from the Food Science and Human Nutrition Department. Panelists were experienced in sensory evaluation techniques (through previous sensory panels on various fruit products, including grape juice), and participated in three training sessions prior to juice evaluations. For training, panelists evaluated muscadine juices with varying muscadine flavor intensities and sweetness levels. They also evaluated juices that had been intentionally 'spoiled' or slightly fermented to illustrate typical off-flavors that may be present. Such off-flavor was generally described as "alcohol or wine-like", but not "acetic-like." The panelists evaluated the juices each week for muscadine flavor intensity, off-flavor level, and sweetness level. Evaluations were on a 15-point scale, and were anchored with 1 = very weak muscadine flavor intensity and 15 = very strong muscadine flavor intensity; 1 = no detectable off-flavor and 15 = high level of off-flavor; 1 = very low sweetness and 15 = very high sweetness. Samples (ca. 30 mL) at 3°C were presented to panelists in wine glasses for evaluation in private booths. The red and white juices were evaluated on different days, and panelists evaluated duplicate samples each week. Data were subjected to analysis of variance using SAS (SAS Institute, Inc., 1985) and analyzed as a split plot over time (Table 1). Data for red and white juices were analyzed separately.

The color of the juice was determined by filtering the sample through a 0.45 μm membrane and then determining the abs. at 420 nm and 520 nm (red juice only) using a spectrophotometer (Perkin Elmer Lambda 3A UV/VIS, Norwalk, CT). A browning index for red juice was calculated as the ratio of abs. at 520 / abs. at 420 nm. The soluble solids were determined with a refractometer (Reichert Abbe Mark II, Reichert Scientific Instruments, Buffalo, NY), pH with a combination electrode (Ross combination, Orion Research, Cambridge, MA) and pH meter (Orion Expandable Ion Analyzer EA920, Orion Research, Cambridge, MA), and titratable acidity by titration with 0.1N NaOH. Acidity was expressed as % tartaric acid. Total plate counts (presumed yeasts) were determined using potato dextrose agar (DIFCO, Detroit, MI) acidified to pH 3.5 with sterile-filtered 0.1% (w/v) tartaric acid. Serial decimal dilutions were made by transferring 1.0 mL aliquots of juice to sterile 0.1% (w/v) peptone diluent until the appropriate dilution range was achieved. Plates were incubated upright at 23 ± 2°C for 5 days.

Samples of juice at each storage time were removed and stored frozen for ethanol analysis. Samples were thawed in water, centrifuged at 9,000 × *g* for 20 min, then filtered through a 0.45 μm nylon filter. A Shimadzu (Kyoto, Japan) GC014A gas chromatograph, a C-R5A recording integrator and an AOC-9 automatic sample injector were used to detect ethanol levels. The column was an Alltech (Deerfield, IL) Econocap Carbowax capillary column, 30 m × 0.32 mm, 1.0 μm film thickness. A 1.0 μL sample was injected. Samples were run isothermally at 32°C with helium linear flow velocity of 25 cm/sec. injector at 275°C and the FID detector at 300°C. Standards of 0.02–0.2% (v/v) ethanol were used for quantitation.

All analyses were performed in duplicate on two replications. All data were subjected to analysis of variance using SAS (SAS Institute, Inc., 1985). Data were analyzed as a factorial with two replications, nine storage times, and two juice treatments (with and without sulfite).

RESULTS & DISCUSSION

THE TREATMENT × TIME INTERACTION was significant for all sensory attributes of the white juice (Table 1). The sensory data indicated that the characteristic muscadine flavor intensity was rather stable for up to 7 wk at 3°C, with a slight decline in flavor in both sulfited and nonsulfited juices through 7 wk (Fig. 1). However, the muscadine flavor intensity of the nonsulfited juice declined after 7 wks, and off-flavor increased (Fig. 2). This off-flavor was described as "alcohol or wine-like." No acetic acid character was noted in this off-flavor. Sweetness levels of the

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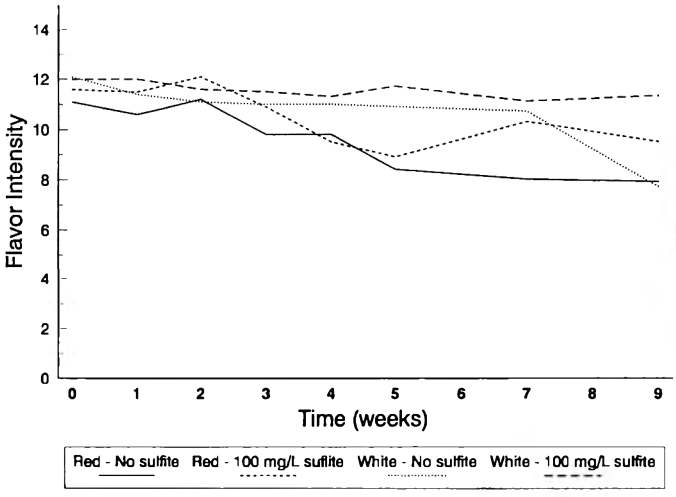

Fig. 1—Effects of storage time at 3°C and sulfite addition on the flavor intensity of red and white muscadine juices, where 1 = very weak muscadine flavor intensity and 15 = very strong muscadine flavor intensity. The storage time × sulfite interaction was significant (0.05 level of probability) for the white juice, but not the red. Refer to Table 1 for the significance of the other main and interactive effects. The standard error ranged from 0.41–0.56 from week to week, with an average of 0.52.

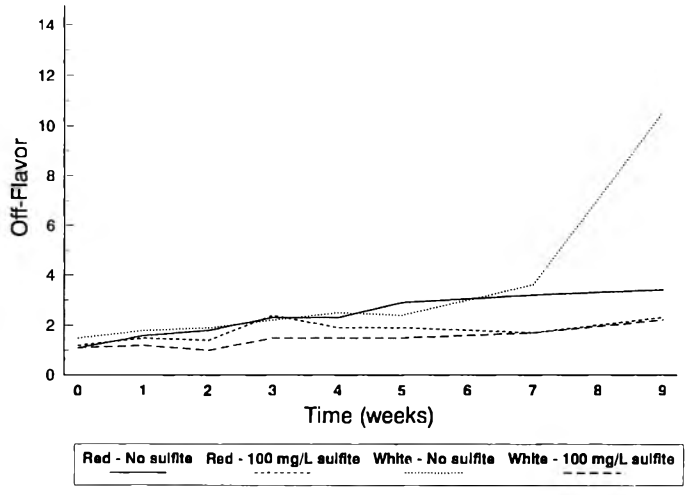

Fig. 2—Effects of storage time at 3°C and sulfite addition on the off-flavor of red and white muscadine juices, where 1 = no detectable off-flavor and 15 = high level of off-flavor. The storage time × sulfite interaction was significant (0.05 level of probability) for the white juice, but not the red. Refer to Table 1 for the significance of the other main and interactive effects. The standard error ranged from 0.15–0.42 from week to week, with an average of 0.35.

Table 1—Analysis of variance model for sensory data and signif cance of F values

Source of variation	Degrees of freedom	Flavor intensity		Off-flavor		Sweetness	
		White juice	Red juice	White juice	Red juice	White juice	Red juice
Replication	1	NS[z]	NS	NS	NS	NS	NS
Panelist	9	*	*	*	*	*	*
Treatment	1	*	*	*	*	*	NS
Panelist × Treatment	9	NS	NS	NS	NS	NS	NS
Panelist × Treatment × Replication	19	NS	NS	NS	NS	NS	NS
Time	7	*	*	*	*	*	NS
Panelists × Time	58	NS	NS	NS	NS	NS	NS
Treatment × Time	7	*	NS	*	NS	*	NS
Panelist × Treatment × Time	58	NS	NS	NS	NS	NS	NS
Error or residual	118						

[z] NS = F value not significant at 0.05, * = F value significant at 0.05. The panelist × treatment interaction was used as the error to test the main effect of treatment. The panelist × time interaction was used as the error to test for the main effect of time. The panelist × treatment × time interaction was used as the error to the treatment × time interaction.

nonsulfited juice began to decline after 7 wk (Fig. 3). In contrast, the sulfited juice had fairly stable flavor intensity and very low off-flavor development through 9 wk at 3°C.

Neither the sulfited or nonsulfited red juice developed notable levels of off-flavors during 9 wk of storage at 3°C, although off-flavor levels increased slightly (significant time main effect), and the sulfited juice tended to have lower off-flavor levels throughout storage (significant main effect of treatment) (Fig. 2). The muscadine flavor intensity of both sulfited and non-sulfited juices decreased during storage (significant main effect of time), but sulfite was effective in limiting the loss of muscadine flavor (significant main effect of treatment) (Fig. 1). The sweetness levels of red juices did not change notably during 9 wk, and no difference in sweetness occurred between sulfited and nonsulfited juices (Fig. 3). No significant treatment × time interactions were found for the sensory attributes of red juice (Table 1).

The ethanol levels in white juices, which usually indicate microbial spoilage and possible off-flavor development, were very low in both sulfited and nonsulfited juices until wk 7, when ethanol levels in nonsulfited juice began to increase (Fig. 4). This increase corresponded to development of off-flavors and loss of muscadine flavor in nonsulfited juice. Sulfited juice continued to have very low ethanol levels through wk 9. The microbial levels (presumed yeast) were consistently higher in the nonsulfited juice during 9 wk, and increased during storage (data not shown). Total plate count values ranged from 4.2–5.2 Log10 CFU/mL in the nonsulfited white juice, and from 3.9–4.0 Log10 CFU/mL in the sulfited white juice. Sulfite obviously lowered microbial levels, and as a result, probably suppressed ethanol and off-flavor production. Previous research has shown that off-flavors in Concord grape juice were noticeable when the ethanol level reached 0.25% (Morris et al., 1979). The ethanol levels in this non-sulfited muscadine juice reached ca. 0.24%, and total plate counts reached >5 log10 CFU/mL. Research on orange juice has shown that many compounds, in addition to ethanol, with low sensory thresholds are produced by yeast during spoilage (Teller, 1993).

Ethanol levels in red juice did not increase notably during 9 wk, even in non-sulfited juice (Fig. 4), which agreed with the lack of off-flavor development in these juices. Microbial levels were higher in non-sulfited juice (4.1–5.4 Log10 CFU/mL) than in sulfited juice (3.5–4.1 Log10 CFU/mL). However, no noticeable signs of microbial spoilage occurred during 9 wk in the nonsulfited juice, although the microbial levels were similar to those in white juice, which did develop signs of microbial spoilage without sulfite.

The color of both sulfited and nonsulfited white juices was similar and very stable during 9 wk at 3°C, showing very little browning during storage (abs. at 420 nm was ≈0.07). Overall, the color of the juices was very good after 9 wk. Muscadine juices lack notable levels of polyphenoloxidase activity (Sims et al., 1991), and since enzymatic browning is not a major problem in these fresh juices, sulfite did little to protect their color. The nonsulfited red juice had much better color (abs. at 520 nm

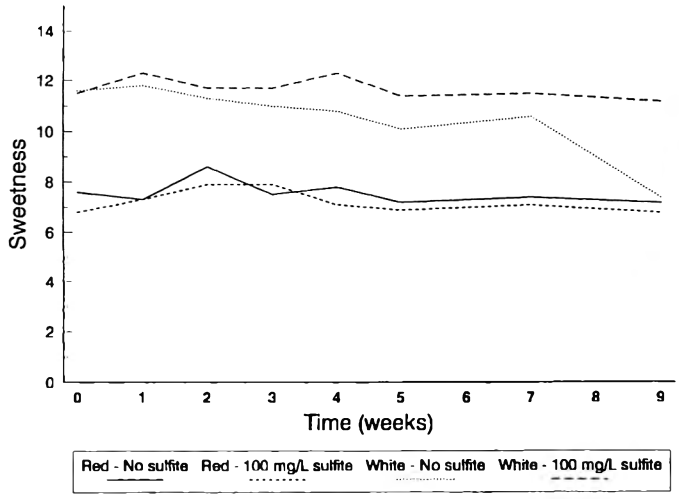

Fig. 3—Effects of storage time at 3°C and sulfite addition on the sweetness level of red and white muscadine juices, where 1 = very low sweetness and 15 = very high sweetness. The storage time × sulfite interaction was significant (0.05 level of probability) for the white juice, but not the red. Refer to Table 1 for the significance of the other main and interactive effects. The standard error ranged from 0.55–0.64 from week to week, with an average of 0.59.

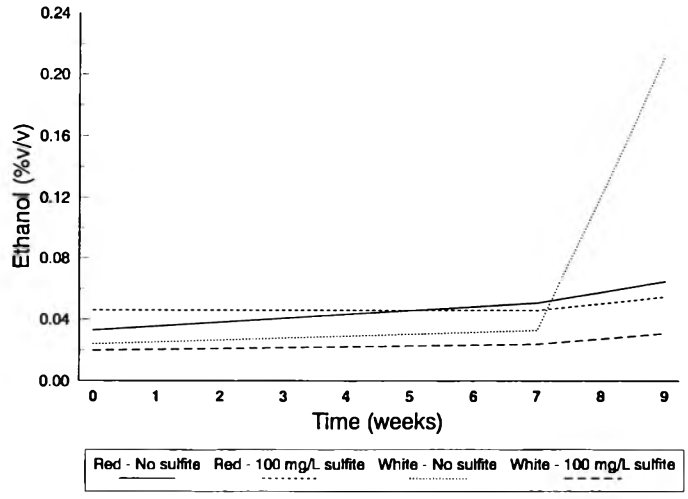

Fig. 4—Effects of storage time at 3°C and sulfite addition on ethanol levels of red and white muscadine juices. The storage time × sulfite interaction was significant (0.05 level of probability) for the white juice, but not the red.

ca. 0.22) than the sulfited juice (abs. at 520 nm ca. 0.12), and did not brown or discolor during 9 wk (data not shown). Sulfite lightened the color of this light red juice, presumably by bleaching anthocyanins (Jurd, 1964), and provided little protection from browning or discoloration. Without polyphenoloxidase activity, the anthocyanins appeared to be stable for this short storage period, and browning was minimal. Although the red color of these cold-pressed juices was rather light, the color of the nonsulfited juice had a pleasant red color which should be very acceptable in this type of juice.

The soluble solids, pH and acidity were about the same for both sulfited and nonsulfited red and white juices, and changed very little during storage (data not shown). Soluble solids averaged 16%, acidity 0.3% (as tartaric), and pH 3.40 for the white juice. Soluble solids of red juices were 13–14%, acidity ≈0.3%, and pH 3.35. Some sediment formed in containers during storage, which probably included some tartrates since juices were not adequately cold stabilized before starting the study.

The overall shelf-life (7–9 wk) of these nonpasteurized juices appeared to be excellent at 3°C. It is not very valid to compare results of this study to the shelf-life of commercial nonpasteurized juices such as orange juice. Nevertheless, the stability of these muscadine juice appeared to be better than the typical 2–3 wk shelf-life of some commercial nonpasteurized juices. The shelf-life of the muscadine juices would have undoubtedly been lower at higher, but perhaps more realistic storage temperatures of 5–10°C. The overall value of sulfite in extending shelf-life of these nonpasteurized juices was minimal at 3°C. Considering that sulfite would have to be declared on the label (over 10 ppm), and the overall negative image of sulfite, its advantages in this type application seem to be questionable.

CONCLUSIONS

NONSULFITED WHITE MUSCADINE JUICE maintained good flavor after 7 wk at 3°C before signs of microbial spoilage became noticeable. Sulfite (100 mg/L) extended the high quality and shelf-life 2 or more wk (through 9 wk). Browning was not a major problem in either sulfited or non-sulfited white juices. Neither sulfited nor non-sulfited red juices developed signs of microbial spoilage during 9 wk, although muscadine flavor decreased during storage, especially in non-sulfited juice. The color of this cold-pressed juice was a pleasant, but light, red. The sulfited red juice had much lighter red color than nonsulfited juice due to anthocyanin bleaching. Overall, with reasonable handling and sanitation and good refrigeration, fresh muscadine grape juice quality can be maintained for at least a month unsulfited and up to 2 mo with 100 mg/L sulfite.

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Survival of *E. coli* and Salmonella after Chilling and Freezing in Liquid Media

M.G. SMITH

ABSTRACT

Predictive microbiology requires a sound basis of observed results, and factors that contribute to death of bacterial cells, before accurate equations can be developed. The effect of chilling and freezing strains of *Escherichia coli* and salmonella serotypes in nutrient broth, a noninhibitory liquid medium, was investigated. The phase of growth, small changes in composition of test medium, and sub-cultures made after primary isolation, influenced survival. Therefore, such influences must be considered when attempting to extrapolate results from pure cultures on laboratory media, to predict behavior of similar organisms in foods during chilling and freezing.

Key Words: chilling, freezing, *E. coli, Salmonella*, predictive microbiology

INTRODUCTION

DEATH OR INJURY OF BACTERIA caused by chilling to temperatures slightly above ice formation (chill shock), or by freezing to <0°C, is an important aspect of refrigerated storage of food (Speck and Cowman, 1969). It is especially important in regard to survival of species of *Enterobacteriaceae* which include food-poisoning organisms such as *Salmonella*, some *Aeromonas* strains, and the enteropathogenic *Escherichia coli*. Reviews (Mazur, 1966; Ingram and Mackay, 1976; MacLeod and Calcott, 1976; Speck and Ray, 1977; Busta, 1978) comprehensively detail our understanding of the effects of such processes.

Reported investigations (Sherman and Cameron, 1934; Hegarty and Weeks, 1940; Meynell, 1958; Strange and Dark, 1962) indicate that actively growing cells are more susceptible to death by chilling and freezing than are those in the stationary phase. The addition of divalent cations, such as magnesium, to liquid media also appeared to enhance survival of bacterial cells after refrigeration (Strange, 1964; Sato and Takahashi, 1968; 1969). Thus, the age of the culture and the composition of the growth medium appear to influence survival. In the developing field of predictive food microbiology (McMeekin and Olley, 1986; Gould, 1989) such effects are very important.

Previous results (Smith, 1985) showed that if the growth of bacterial cells, such as *E. coli* SF, *S. typhirmurium* 55 and "wild-type" coliform organisms, could be measured accurately in blended meat, mathematical equations could be developed to represent the lag and log phases of growth. These could be used subsequently to calculate the expected increase of such bacteria under different, even constantly changing, conditions (Smith, 1987). Those results have been used to develop other mathematical models to represent the growth of such bacteria (Davey, 1991; Ratkowsky et al., 1991). For further progress in this field, knowledge of the effects of chilling and freezing on such bacteria in the lag and log phases of growth and in the stationary phase, was needed.

The research objectives were to determine the effects of chilling and freezing pure cultures of *E. coli* strains and different *Salmonella* serotypes, in either the lag or log phases of growth, and in the stationary phase, in liquid media.

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MATERIALS & METHODS

Bacterial cultures

Strain *E. coli* SF was originally isolated from sheep feces. It could be readily differentiated from other strains as it fermented both amygdalin and saccharose, an unusual combination of characteristics found in less than 0.1% of *E. coli* organisms (API Systems S.A., La Balme Les Grottes, France). The *S. typhimurium* 55 culture was originally isolated from cattle feces. The two strains had been maintained, with numerous subcultures, on Oxoid nutrient agar slopes kept at 0–2°C for at least 10 yr.

The wild-type *E. coli* strains were isolated from abattoir effluent and identified using API 20E strips. The 20 different *Salmonella* serotypes were originally isolated from a variety of sources (sheep and cattle feces, holding yards, and abattoir effluent) and maintained on nutrient agar (NA) slopes at 0–2°C. The serotypes were identified by the Salmonella Reference Centre, Adelaide, Australia.

Media

The liquid growth media were Oxoid nutrient broth alone (NB) and NB to which 5×10^{-3}M $MgSO_4$ was added (NB Mg^{++}) to provide an excess of magnesium ions. Counts of *E. coli* were made on two media, Oxoid MacConkey agar No.3 (MAC) and Oxoid Tryptone Soya agar to which 2g of glucose and 2g of Oxoid yeast extract were added/L (TYSG). Salmonellae were counted on Oxoid Brilliant Green agar (BGA), specially made less inhibitory by adjusting to pH 7.2, and on TYSG. All counts were made by surface inoculation on pre-dried plates.

Experimental technique

Each bacterial culture was inoculated into 10 mL of NB and incubated at 37°C for 24 hr to give $\approx 10^8$ cells/mL in the stationary phase of growth. This was diluted 1000-fold into 100 mL chilled (0–2°C) growth media (NB or NB Mg^{++}) and 1 mL aliquots were distributed into the required number of pre-chilled 150 × 15 mm sterile test tubes covered with loose aluminum caps. The tubes were kept chilled (0–2°C overnight) and then placed in a circulating water bath at 25 ± 0.1°C. Until this time, no growth would have taken place because of the low temperature (Smith, 1985).

To obtain the initial or zero time number of cells present, counts were made on two tubes, selected at random, after about 5 min incubation in the water bath. At the same time, 2 tubes were placed in ice water (by definition 0°C) and another two tubes were placed in a freezer at −30°C. Tests showed the temperature of the liquid media fell from 25°C to 0°C in the ice water, or froze solid in the freezer, within 10 min. After 24 hr the frozen media was allowed to thaw (30 min at 20°C) and counts were made on those tubes and on the tubes kept in ice water. Dilutions were made in 0.1% (w/v) Oxoid peptone water. The plates were incubated at 37°C for 18–24 hr. Results were calculated as means of the $\log_{10}$ CFU (colony forming units)/mL (Tanner, 1944).

For growth rates, the required number of tubes were prepared and incubated in the water bath at 25 ± 0.1°C. At the designated intervals, counts were made on two tubes. At the same time, two tubes were placed in ice water and another two tubes were placed in the freezer. Counts were made subsequently on these after 24 hr.

The *E. coli* and *Salmonella* organisms that were to be treated in a stationary phase of growth were first incubated in NB at 37°C for 72 hr. They were then diluted 1000-fold into 100 mL of chilled (0–2°C) media (NB or NB Mg^{++}) and 1 mL aliquots were distributed into the required number of pre-chilled 150 × 15mm sterile test tubes covered with loose aluminum caps. The tubes were kept chilled (0–2°C) overnight and then placed in a circulating water bath at 25 ± 0.1°C for about 5 min, just sufficient time to ensure they had reached the required temperature. When they were to be treated in a log phase of growth, the tubes were first incubated in a water bath at 25°C for 4 hr to ensure the bacterial

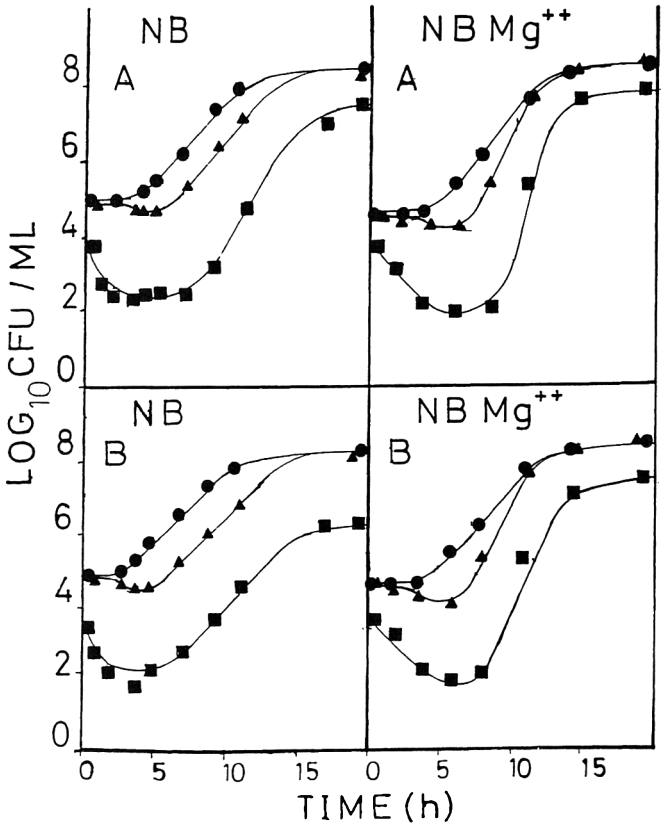

Fig. 1—Growth (●), chill death (▲) and death on freezing (■), in nutrient broth (NB), and nutrient broth containing an excess of magnesium ions (NB Mg^{++}), of *E. coli* SF (A), and *S. typhimurium* 55 (B), counted on TYSG agar.

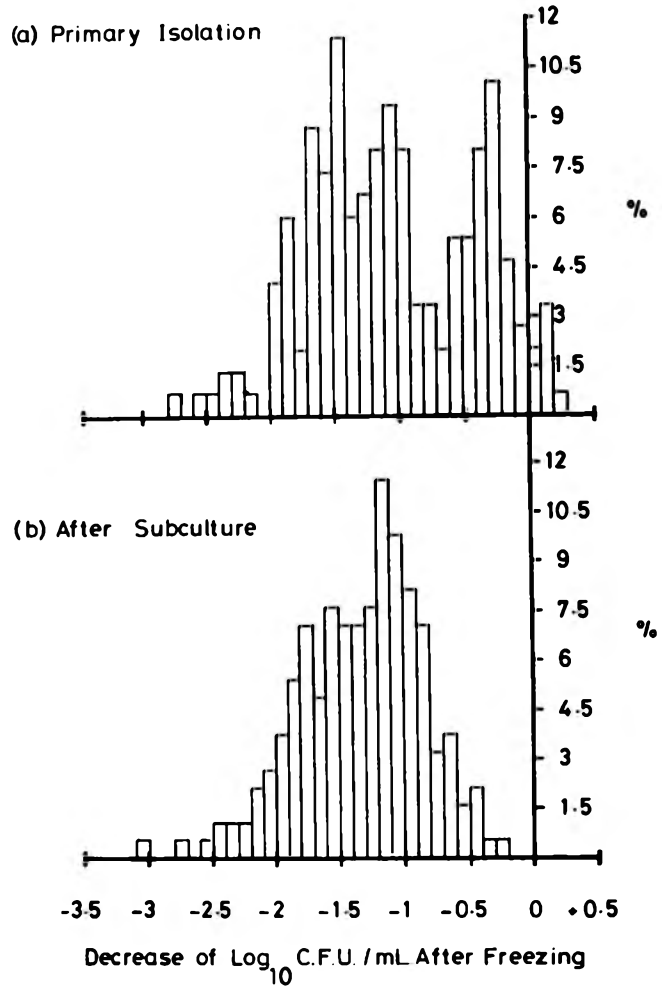

Fig. 2—Histogram of the decrease of log_{10} CFU/mL of 200 *E. coli* strains frozen in the stationary phase in NB and counted on MAC agar after isolation and after subculture and storage.

Table 1—Means of log_{10} CFU/mL of 10 replicates each of *E. coli* SF and *S. typhimurium* 55 in the stationary phase of growth counted on selective (MAC or BGA) and nonselective (TYSG) agar plates

Organism	Media		Significance (t test)
	MAC or BGA	TYSG	
E. coli SF	5.34 (0.12)[a]	5.49 (0.10)	NS
S. typhimurium 55	5.54 (0.13)	5.55 (0.12)	NS

[a] ( ) Standard deviation; NS = Not Significant.

Table 2—Decrease of Log_{10} CFU/mL of 20 strains of *E. coli* and 20 *Salmonella* serotypes chilled or frozen in duplicate in either the early log or the stationary phase of growth in NB and NB Mg^{++}

Growth phase	Treatment	Organisms	NB	NB Mg^{++}	Significance (t test)
MAC or BGA					
Log	Chilled	*E. coli* strains	−1.34 (0.39)[a]	−0.88 (0.33)	**
		Salmonellas	−1.35 (0.35)	−0.83 (0.35)	**
	Frozen	*E. coli* strains	−4.68 (0.44)	−3.21 (0.46)	***
		Salmonellas	−3.34 (0.53)	−2.68 (0.35)	***
Stationary	Frozen	*E. coli* strains	−1.29 (0.23)	−0.90 (0.26)	**
		Salmonellas	−1.45 (0.21)	−0.87 (0.30)	**
TYSG					
Log	Chilled	*E. coli* strains	−1.33 (0.14)	−0.87 (0.34)	**
		Salmonellas	−1.21 (0.22)	−0.74 (0.14)	**
	Frozen	*E. coli* strains	−4.47 (0.54)	−2.92 (0.37)	***
		Salmonellas	−3.31 (0.52)	−2.49 (0.34)	***
Stationary	Frozen	*E. coli* strains	−1.24 (0.15)	−0.88 (0.31)	**
		Salmonellas	−1.38 (0.32)	−0.81 (0.18)	**

[a] ( ) Standard deviation; test NB ~ NBMg++
** $P < 0.01$
*** $P < 0.001$

cells were in an early log phase of growth (Smith, 1985). The diluting medium (0.1% peptone water) was always adjusted to room temperature (20°C) before use to avoid any effects due to cold-shock on bacterial cells.

RESULTS & DISCUSSION

THE SELECTIVE PLATING MEDIA (MAC or BGA) were compared with nonselective medium (TYSG) using the test organisms, *E. coli* SF and *S. typhimurium* 55, diluted 1000-fold after they had been grown to the stationary phase (72 hr at 37°C) in NB (Table 1). No statistical differences were found between counts on selective and nonselective media. Therefore, any difference observed in the experiments must have been due either to the phase of growth, or the media in which the organisms were grown (NB or NB Mg^{++}), or to temperature changes to which the organisms were subjected.

Growth at 25°C in NB and NB Mg^{++}, the decrease after chilling at 0°C, and freezing at −30°C for 24 hr, on TYSG were compared (Fig. 1). The generation times (G = 0.75 h) of the *E. coli* SF and *S. typhimurium* 55 in both NB and NB Mg^{++} media were the same as reported in blended meat tissue (Smith, 1985).

As the cells began to metabolize on incubation at 25°C, they apparently became progressively susceptible to the lethal action of chilling even before cell division had taken place, i.e. before the growth curve began to rise. They became more susceptible to this effect in the late lag phase and were most susceptible during the exponential (log) phase. The proportion of cells in each culture that were nonviable after chilling in NB Mg^{++} was

Table 3—Fit of mixture of two normal distributions to histograms in (a) of Fig. 2 and 3, and single normal distribution to histograms in (b) of Fig. 2 and 3

Treatment	Media	Distribution 1 Mean	Distribution 1 S.D.[a]	Distribution 2 Mean	Distribution 2 S.D.	Percentage in first population (Distribution 1)	x^2 (4df)	Significance
Primary isolation	MAC	−1.33	0.22	−0.24	0.05	73%	26.7	***
	TYSG	−0.92	0.27	−0.47	0.08	19%	20.5	***
After subculture	MAC	−1.35	0.48					
	TYSG	−0.73	0.39					

[a] S.D. Standard deviation
*** $P < 0.001$.

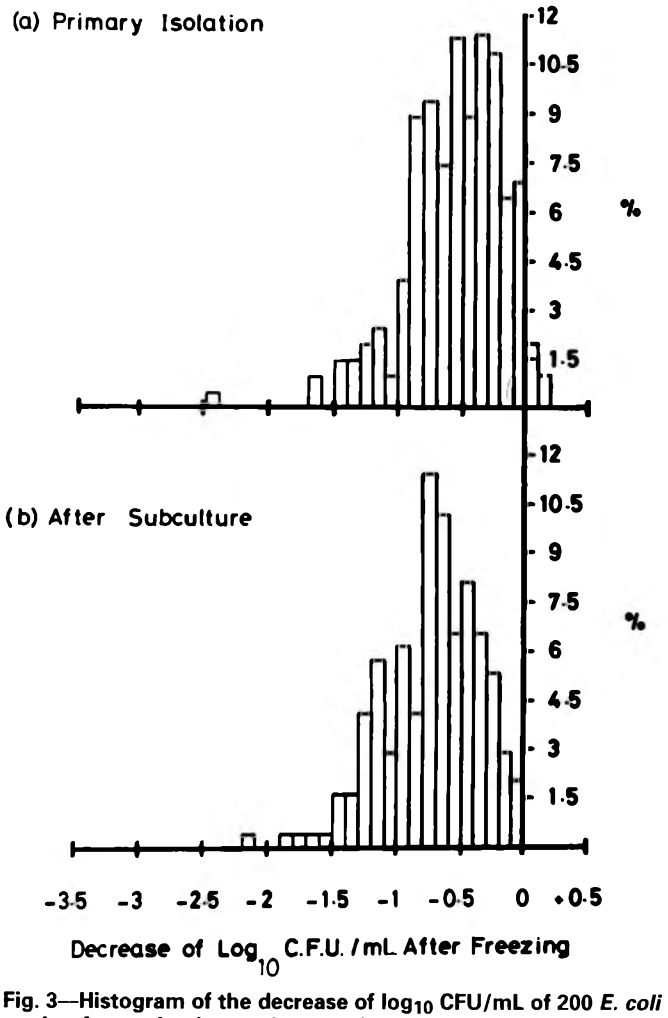

Fig. 3—Histogram of the decrease of $\log_{10}$ CFU/mL of 200 *E. coli* strains frozen in the stationary phase in NB and counted on TYSG agar after isolation and after subculture and storage.

similar to that of NB, except in the late log phase when they appeared to become progressively more resistant to the effect. Thus, the addition of Mg ions had a protective effect in liquid media but this only became important when large numbers of organisms were present ($\approx 10^6$ cells/mL) in the later stages of the log phase. However, as normal Oxoid NB contains some magnesium ions, (Anonymous, 1976) this effect may be different in other growth media. The composition of the media, including small quantities of at least one metal ion, could apparently be important in the survival of these bacterial strains after chilling. The same phenomenon would probably occur in foodstuffs.

Freezing (24 hr at −30°C) had a more marked effect on the test organisms at all stages of the growth cycle (Fig. 1), but the organisms were most susceptible during the late lag and early log phases. The addition of extra magnesium ions to the NB media changed the shape of the survivor curves to some extent

Table 4—Mean decrease of $\log_{10}$ CFU/mL of five strains of *E. coli* frozen (five replicates/strain) ir the stationary phase in nutrient broth

Treatment	MAC	TYSG
Primary isolation (P)	0.22 (0.14)[a]	0.25 (0.15)
After 28 days incubation (I)	0.18 (0.13)	0.19 (0.21)
After subculture (S)	1.40 (0.28)	0.25 (0.18)
(P v I)[b]	NS	NS
(P v S)[b]	***	NS

[a] ( ) Standard deviation; NS = Not significant
[b] Test of difference in means
*** $P < 0.001$.

Table 5—Mean decrease of $\log_{10}$ CFU/mL of 10 replicates of each of 10 strains of *E. coli* and of each of two strains of two *Salmonella* serotypes frozen in the stationary phase in NB and counted on TYSG

Organism	Decrease	Organism	Decrease	Significance (t test)
E. coli B22	−0.13 (0.03)[a]	*E. coli* B148	−1.24 (0.22)	***
E. coli B25	−0.03 (0.15)	*E. coli* B167	−1.29 (0.16)	***
E. coli B30	−0.12 (0.07)	*E. coli* C1	−1.22 (0.21)	***
E. coli B46	−0.10 (0.18)	*E. coli* C3	−1.21 (0.19)	***
E. coli B48	−0.23 (0.01)	*E. coli* B27	−1.57 (0.17)	***
S. anatum Strain 1	−1.03 (0.15)	*S. anatum* Strain 2	−1.72 (0.17)	***
S. typhimurium Strain 1	−1.12 (0.18)	*S. typhimurium* Strain 2	−1.80 (0.15)	

[a] ( ) Standard deviation
*** $P < 0.001$.

especially in the later stages of log growth. Again, results indicated that the composition of the growth medium (or of a foodstuff) could affect the proportion of cells which survived freezing.

To investigate these effects further, 20 *E. coli* strains and 20 *Salmonella* serotypes were chilled or frozen in the log and stationary phases of growth in either NB or NB Mg^{++} (Table 2). All cultures had been stored previously for several years on NA slopes at 0–2°C and each had been sub-inoculated several times. Results indicated that the effects of chilling and freezing (Fig. 1) on the two test strains, *E. coli* SF and *S. typhimurium* 55, were also shown on other strains of these genera. Addition of extra magnesium ions to the medium also obviously conferred a notable protective effect on the cells ($P < 0.01$).

The death of the organisms due to chilling could only be a function of the change of temperature as no ice formed. Thus, no physical disruption of the surface membranes occurred although some chemical change may have taken place in the lipid layer. Nevertheless, any such change should have been readily reversed when cells were again incubated at 25°C and should not have resulted in any permanent physical damage. The greater effect due to freezing was most probably due to ice formation both outside and within the cells (Mazur, 1966; Ingram and Mackey, 1976; Macleod and Calcott, 1976).

A more detailed investigation was undertaken to examine the effect of freezing wild-type *E. coli* strains in NB. The results on 200 *E. coli* strains isolated from abattoir effluents were compared (Fig. 2, 3).

On primary isolation these *E. coli* strains appeared to consist of at least 2 distinct populations in their resistance to freezing death. Statistical analysis using the method of McLachlan and Jones (1988, Table 3), showed that the fit of two distributions to the results obtained with primary isolates was better ($P < 0.001$) than regarding the organisms as a heterogeneous population that varies (albeit widely) about some mean value. After sub-culture and storage 14 days at 0–2°C on NA slopes, the same *E. coli* strains were tested again. Only one distribution could then be shown by the same statistical analysis (Table 3). This could be explained by the rapid loss of highly labile plasmids from some of the wild-type strains when cultured and stored on laboratory media or there may have been some other rapid and irreversible alteration in the genetic characteristics of the cells. This demonstrated again that care must be taken before attempting to extrapolate the degree of freezing death of *E. coli* strains from laboratory results to estimate how such organisms would behave in foodstuffs.

The effect observed from Table 3 was investigated further. Another 25 wild-type *E. coli* strains were isolated from abattoir effluent. Five were highly resistant to the lethal action of freezing in NB. These five were tested before and after sub-culture on NA slopes (14 days at 0–2°C) and also the original NB culture was tested again after 28 days at room temperature (Table 4). Results showed a highly significant effect ($P < 0.001$) due to the sub-culture and storage of these five strains on NA slopes when plate counts were made on MAC agar. No effect was found when TYSG was plating medium, nor was an effect shown on MAC agar after extended incubation (28 days) in NB.

Other experiments showed there could be a significant difference ($P < 0.001$) after freezing in NB in the decreases between *E. coli* strains when counts were made on TYSG agar. The strains had been intentionally selected to demonstrate large differences. Such differences could also occur between different strains of the same *Salmonella* serotypes (Table 5).

In the same way, organisms could be selected to demonstrate no significant differences in decrease after freezing, whether the decrease was small ($<\log_{10}$ 0.2) or large ($>\log_{10}$ 1.0), (Table 5). Therefore, the susceptibility to death by freezing in liquid media such as NB is a characteristic of a particular strain. The results using any one strain, whether of *E. coli* or of *Salmonella* would not necessarily predict the response shown by other strains.

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Preparation of Alkyl Esters of Pectin and Pectic Acid

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ABSTRACT

Alkyl esters of pectin and pectic acid have been prepared via three different synthetic routes. Enhancements of intrinsic viscosity, binding to bile acids and to isolated soy protein were shown for these novel pectin derivatives. The development of new and improved pectin-containing products may be enhanced by the ease of preparation and improved functionality of such compounds.

Key Words: pectin, pectic acid, alkyl esters, esters, hydrophobic

INTRODUCTION

PECTIN IS A POLYMER of partially methoxylated α-1,4-linked D-galacturonic acid interrupted by dispersed or alternating 1,2-linked L-rhamnose units which are subsequently linked to neutral sugars, primarily D-galactose and L-arabinose. Pectin is an abundant and ubiquitous structural component of all dicotyledonous and most monocotyledonous plants. Obtained primarily from citrus peels and apple pomace, it has major industrial use as a gelling agent in the manufacture of jams and jellies.

Pectins may be classified according to their degree of methoxylation (DM). Those with DM >50% are high-methoxyl pectins and those with DM <50% are low-methoxyl pectins. By altering the DM, pectins with a broad range of hydrodynamic properties may be prepared and are commercially available. Amidated pectins, useful in the manufacture of low-sugar jams and jellies are also available. Pectic acid sulphates have been prepared (Alburn and Seifter, 1956; Schweiger and Andrew, 1972) and have high anticoagulant activity, analagous to heparin, and low toxicity (Alburn and Seifter, 1956). The chemical modification of pectin is potentially facilitated by its complexity and diversity of functional groups. It is also easily subject to degradation via a non-enzymatic trans elimination reaction, even in neutral solution (Albersheim et al., 1960) and also via reactions of enzymes such as pectinmethylesterase and polygalacturonase.

The solution behavior of pectin is influenced by several factors: hydrogen bonding, ionic character and hydrophobic character. Hydrogen bonding is favored by a pH < pK_a. Ionic character is dependent upon free carboxyl content and the presence of cations, and is favored by high water activity and by a pH > pK_a. Charge-charge repulsion as well as the presence of neutral side chains are important in the prevention of intermolecular association among pectin molecules. Hydrophobic character is dependent upon the methyl ester content and is favored by low water activity.

Pectin has been implicated in the modulation of serum cholestrol through a negative feedback system within the enterohepatic cycle (Pandolf and Clydesdale, 1992; Pfeffer et al., 1981). Pectin binds with bile acids, which are catabolic products of cholestrol. The nature of this binding is complex and may involve low molecular weight pectins (Pfeffer et al., 1981), high molecular weight/high methoxy pectin (Judd et al., 1977) and may also involve ionic complexes with minerals (Pandolf and Clydesdale, 1992). The preparation of alkyl esters of pectin and pectic acid and measurement of their binding constants with bile acids enables examining the contribution of hydrophobic binding exclusive of other variables.

By adding to or replacing methyl esters with higher alkyl esters the hydrophobic properties of pectin may be further exploited and products of greater utility and value may be developed. Expanding the uses of pectin within the food and pharmaceutical industries could greatly increase demand for pectin. Our objective was to prepare such novel pectin esters (PCAEs) and pectic acid alkyl esters (PAAEs).

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MATERIALS & METHODS

Materials

Citrus pectin (P 9135), cholic acid (C-1129), glycocholic acid (G-2878) and 3° Molecular Sieves (M 9882) were purchased (Sigma Chemical Co. St. Louis, MO). The citrus pectin had an inherent 76% degree of esterification. Triethyl orthoacetate, 1-alkyl-3-nitro-1-nitrosoguanidines and p-toluenesulphonic acid monohydrate (pTSA) were purchased (Aldrich Chemical Co. Inc. Milwaulee, WI). Isolated Soy Protein (PP 710) was donated by Protein Technologies International (PTI, Checkerboard Square, St. Louis, MO). All reagents were of the highest purity obtainable.

Preparation of pectin and pectic acid

A 1% solution of citrus pectin was prepared by overnight dissolution in water at room temperature (20°–25°C) with magnetic stirring. The solution was heated in a water bath at 95°C for 0, 30 or 60 min. to effect partial hydrolysis via trans elimination. To prepare pectic acid, to the 1% pectin solution sufficient 5N KOH was added to yield 0.5N KOH. This solution was stirred at room temperature for 30 min. The resultant pectate solution was then titrated to pH 3.5–4.0 with conc. phosphoric acid. The pectic acid/potassium phosphate solution was then extensively dialyzed vs water at 4°C in a Spectra/Por 3 dialysis membrane tubing of MWCO 3,500 (Spectrum Medical Industries, Los Angeles, CA) to remove phosphate salts.

For either the pectin or pectic acid solutions, three volumes of 95% ethanol were added, and the suspension stirred magnetically, to effect precipitation. The precipitate was isolated via vacuum filtration, resuspended in 100% ethanol and again isolated. This extraction procedure was repeated with acetone and the product resuspended in dichloromethane for Methods 1 and 2 or ether for Method 3 (described below). For Methods DZA and TOA, the pectin or pectic acid remained suspended in dichloromethane. For Method ATS, the precipitate was vacuum filtered and the residual solvents removed under high vacuum. The resulting pectin or pectic acid thus obtained through final extraction in dichloromethane was swollen and remained suspended. The pectin or pectic acid obtained via ether extraction and subsequent drying were sufficiently dehydrated and markedly less dense than the commercial starting material. These dehydration and extraction steps were necessary for effective synthesis of the alkyl esters as their extended conformation presumably diminished steric hindrance.

Synthesis of pectin and pectic acid alkyl esters
Diazoalkane (DZA) method

Diazoalkanes were prepared in ether via the corresponding 1-alkyl-3-nitro-1-nitrosoguanidine according to the filtration method of McKay et al. (1950). A yield of 50% of each diazoalkane was assumed. Based on the assumption that 1g pectic acid contains 5.68 mmol -COOH groups, at least a threefold molar excess of the diazoalkane was used. The pectin or pectic acid was esterified by a modification of the diazomethane procedure of Deuel et al. (1950). Accordingly, pectin or pectic acid, (2g) suspended in about 200 mL dichloromethane as previously described, was placed in an oversized (500mL) three-necked reaction flask. The

suspension was chilled to −23°C by the slow, careful addition of small pieces of dry ice directly to the reaction mixture and constant magnetic stirring. The diazoalkane (in ether) was added dropwise, with constant stirring, via an addition funnel while maintaining the temperature between −23° and −25°C. 1-Alkyl-3-nitro-1-nitrosoguanidines are hazardous materials; they are explosive, mutagenic solids. Their resulting diazoalkanes in ether are explosive. Great care must be used in working with these materials in order to minimize their potential hazards. Rubber gloves and a respirator should be worn while handling 1-alkyl-3-nitro-1-nitrosoguanidines. Etherial solutions of diazoalkanes must be stored in glassware without ground glass joints below 0°C. All procedures must be carried out in a well ventilated fume hood. The resulting PCAE or PAAE was isolated via vacuum filtration and washed with ethanol prechilled to −23°C, followed by ethanol, acetone and ether at room temperature. The final product was dried in vacuum and stored at 4°C for future use. Glacial acetic acid was added to the dichloromethane reaction filtrate to destroy any residual diazoalkane, which changed from yellow to colorless.

Triethyl orthoacetate (TOA) method

A modification of the triethyl orthoacetate procedure of Trujillo and Gopalan (1993) was used. Pectic acid, (1g) suspended in about 100 mL dichloromethane as previously described, was placed in a 250 mL single-necked reaction flask. A tenfold molar excess of triethyl orthoacetate (10.4 mL) was added. The suspension was stirred magnetically at room temperature for 3 days. The resulting PAAE was isolated via vacuum filtration, washed with ethanol, acetone and ether. The final product was dried in vacuum and stored at 4°C for future use.

Alkanol/p-toluenesulphonic acid (ATS) method

Dried pectin or pectic acid as previously described (1g) was added to about 100 mL of the appropriate alkanol (methanol, ethanol, etc.) along with 1g pTSA and 2g 3Å molecular sieve. The suspension was stirred magnetically at room temperature for 3 days. An additional 1g molecular sieve was added after 1 day and again after 2 days. The suspension was vacuum filtered and washed several times with the same alkanol. The air-dried residue which contained the PCAE or PAAE along with the molecular sieve was suspended in about 100 mL water and stirred magnetically for several hours to overnight. The PCAE or PAAE, which dissolved completely, was separated from the molecular sieve by vacuum filtration, precipitated with 95% ethanol, washed with ethanol, acetone and finally with ether. The final product was dried in vacuum and stored at 4°C for future use.

^{1}H NMR

Qualitative confirmation of esterification was established by ^{1}H NMR at 270 MHz in D_2O at 35°C. Chemical shifts were referenced to dioxane as an external standard (∂ 3.77).

Determination of degree of esterification

Degree of methyl esterification was determined by the method of Klavons and Bennett (1986). The degree of C-2 and higher esterification was determined by the Sigma Diagnostics Alcohol assay kit (#332-A) utilizing alcohol dehydrogenase (Sigma Chemical Co. St. Louis, MO) after hydrolysis of the PCAEs or PAAEs with 0.5N KOH as described previously.

Determination of intrinsic viscosity

Flow times of the PCAEs and PAAEs were measured in a Cannon-Ubbelohde Semi-Micro Type Viscometer with a constant of 0.00833 mm^2/s^2. The solvent was 0.155N NaCl. Intrinsic viscosities, [η], were calculated according to the method of Kravtchenko and Pilnik (1989):

$$[\eta] = 1/C[2(\eta/\eta_o - 1 - \ln\eta/\eta_\infty)]^{1/2} \quad (1)$$

The molecular weights (M_w) of the original pectin, the 30 min hydrolyzed pectin and the 60 min hydrolyzed pectin were estimated by the Mark-Houwink equation of Chou et al. (1991) for pectins with M_w below 100,000:

$$[\eta] = 9.55 \times 10^{-2} M_w^{0.73} \quad (2)$$

Equilibrium dialysis

Equilibrium dialysis experiments were conducted with solutions of cholic acid or glycocholic acid at concentrations of 0.25 mg/mL Krebs-Ringer phosphate buffer, pH 6.3 in one half of a five-chamber dialysis cell (Bel-Art Scienceware, Pequannock, NJ) vs a solution of the PAAEs/PCAEs at 10 mg/mL Krebs-Ringer phosphate buffer, pH 6.3 according to Pfeffer et al. (1981). Each half-cell had a volume of 1.0 mL, a surface area of 2.63 cm^2 and a Q factor (ratio of surface area to half-cell volume) of 2.63 cm^{-1}. The half-cells were separated by Spectra-Por 1 dialysis membrane tubing of MWCO 6,000–8,000 (Spectrum Medical Industries, Los Angeles, CA). The cell was placed on a platform shaker at 23°C for 2 days. Bile acids were determined with the Sigma Diagnostics Bile Acids assay kit (#450-A), (Sigma Chemical Co. St. Louis, MO).

Binding of PAAEs to isolated soy protein

A 1% solution of solubilized isolated soy protein (ISP) was prepared in 20 mM potassium bicarbonate buffer, pH 11.0 as described in Klavons et al. (1992). A 1% solution of pectic acid or pectic acid ethyl ester was prepared in 1.11% citric acid, pH 3.7. A 1 mL aliquot of ISP was added to 9 mL of either pectic acid or pectic acid ethyl ester. The resulting precipitate (in 1% citric acid, pH 3.7) was isolated via centrifugation, washed with water and solubilized in 0.05N KOH as described in Klavons et al. (1992). Protein was determined by the method of Lowry et al. (1951). Pectin was determined by the method of McComb and McCready (1952).

RESULTS & DISCUSSION

THE M_w of the original pectin, the 30 min hydrolyzed pectin, and the 60 min hydrolyzed pectin were 76.7, 57.2 and 51.2 kDa, respectively, as calculated by Eq. (2). The methoxylated pectin showed a characteristic methyl ester singlet at ∂ 3.82 in the proton NMR spectrum. Likewise, in the spectrum of the ethoxylated pectin signals characteristic of an ethyl ester (methyl triplet at ∂ 1.30 and oxymethylene quartet at ∂ 4.28) were observed. In the case of the propoxylated pectin a methyl triplet at ∂ 0.94, a methylene quartet at ∂ 1.74 and an oxymethylene triplet at ∂ 4.17 showed the presence of a propyl ester.

The efficiencies of pectin esterification were compared (Table 1) and those of pectic acid (Table 2). Partial hydrolysis of the polygalacturonan backbone facilitates synthesis of the PCAEs/PAAEs, presumably due to diminished steric hinderance. The decrease in M_w effects a decrease in the intrinsic viscosity (Table 3); however, the incorporation of higher esters effects an increase. This suggests an extended conformation (unfolding) of the backbone of the PCAEs and decrease of hydrodynamic interaction (Chou et al., 1991).

Table 1—Efficiency of esterification of pectin

Prior heating	Synthesis[a]	Alkyl group	% Esterification effected
None	ATS	Methyl	89.5
60 min	ATS	Methyl	87.9
None	ATS	Ethyl	20.5
30 min	ATS	Ethyl	24.4
60 min	ATS	Ethyl	27.6
60 min	TOA	Ethyl	11.3

[a] ATS = alkanol/p-toluenesulfonic acid; TOA = Triethyl orthoacetate.

Table 2—Efficiency of esterification of pectin acid (60 min prior heating)

Synthesis[a]	Alkyl group	% Esterification effected
DZA	Methyl	100
ATS	Methyl	100
DZA	Ethyl	23.4
ATS	Ethyl	8.9
DZA	Propyl	18.9
ATS	Propyl	8.4
DZA	Butyl	4
DZA	Amyl	10
DZA	Hexyl	5

[a] ATS = alkanal/p-toluenesulfonic acid; DZA = Diazoalkane

Table 3—Intrinsic viscosities of pectins

Prior Heating	Synthesis[a]	Alkyl Group(s)	η (mL/g)
None	None	Original pectin (76% Methoxy)	351.4
30 min	None	Original pectin (76% Methoxy)	283.7
60 min	None	Original pectin (76% Methoxy)	261.6
None	ATS	Original pectin (90% Methoxy)	394.5
60 min	DZA	Original pectin (100% Methoxy)	406.1
None	ATS	Original pectin (76% Methoxy, 21% Ethoxy)	423.2
30 min	ATS	Original pectin (76% Methoxy, 24% Ethoxy)	381.6
None	ATS	Original pectin (76% Methoxy, 2.2% Propoxy)	417.8
30 min	ATS	Original pectin (76% Methoxy, 2.4% Propoxy)	382.6

[a] See Table 1, 2 for codes.

Equilibrium constants for binding of the PCAEs/PAAEs to bile acids were compared (Table 4). The equilibrium constants for binding of glycocholic acid to the PCAEs/PAAEs increase with increasing hydrophobic character (higher alkyl groups). Likewise, no binding of cholic acid to unaltered pectin was observed; however, cholic acid binds to pectic acid ethyl ester. Pectin appears to be related to the modulation of serum cholesterol levels via negative feedback in the enterohepatic cycle. Other factors such as hydrogen bonding, ionic character and mineral supplementation likely are also important. Clearly a contribution comes from the hydrophobic character in the binding of bile acids to pectin. The PCAEs/PAAEs may be more effective in this respect due to increased hydrophobic binding capacity.

We have demonstrated the effect pectin has in citrus cloud formation and stability and discussed the importance of the hydrophobic character (Klavons et al., 1994a). These effects were applied to prepare a beverage clouding agent from pectin and isolated soy protein (Klavons et al., 1994b). The binding of PAAEs to ISP was measured (Table 5). The pectic acid ethyl ester showed a three-fold increase in binding with the ISP over the equivalent amount of nonesterified pectic acid. This binding may be attributed to the increase in hydrophobic character of the ester. The enhanced hydrophobic binding to proteins may also be useful for development of emulsions, stabilizers or beverage clouding agents.

The ease of preparation (via the ATS method) and enhanced physicochemical properties of the PCAEs and PAAEs could create new uses and markets for pectins and existing pectin-containing commodities. Baldwin and Pressey (1988) demonstrated the ability of pectin oligomers to stimulate production of ethylene in tomato fruit via treatment with polygalacturonase (PG) and pectin methyl esterase (PME). Oligomers of PCAEs/PAAEs may also serve as elicitors of ethylene or phytoalexins, or as inhibitors of pectin methyl esterase.

Table 4—Equilibrium binding constants of pectins and pectic acid with bile acids

Pectin type	Heating time (min)	Bile acid	K (M^{-1})
Pectic Acid	60	Glycocholic	no binding
Pectin (76% ME)	none	Cholic	no binding
Pectin (76% ME)	60	Glycocholic	1.1×10^3
Pectin Methyl Ester	60	Glycocholic	3.3×10^3
Pectin Ethyl Ester	60	Glycocholic	1.4×10^4
Pectin Propyl Ester	60	Glycocholic	4.2×10^4
Pectin Ethyl Ester	60	Cholic	4.1×10^4

Table 5—Binding of PAAEs to isolated soy protein (60 min prior heating)

Pectin Type	[Pectin] / [Protein]
Pectic acid	0.72
Pectic acid ethyl ester (23.4%)	2.19

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Branched Oligosaccharides Concentrated by Yeast Fermentation and Effectiveness as a Low Sweetness Humectant

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ABSTRACT

A branched oligosaccharides (BOS) mixture was prepared from liquefied corn starch using *Bacillus licheniformis* maltogenic amylase (BLMA). A highly concentrated BOS was prepared by removing glucose and maltose through yeast fermentation, which increased the BOS content. Water activity (a_w) of bread went from 0.87 to 0.82 when 10% (w/w) or to 0.78 when 20% (w/w) BOS was added. BOS lowered a_w more effectively as concentration of BOS increased. The water sorption isotherm of BOS was much higher than that of sucrose at a_w, 0.1–0.8. BOS also prevented starch retrogradation in bread, probably due to steric hinderance and to the state of water retained by BOS. Relative sweetness of BOS was ≈17.5% of sucrose. The BOS mixture produced by BLMA might provide a new type humectant for foods that is low in sweetness and retards retrogradation.

Key Words: oligosaccharides, yeast fermentation, humectant, water activity, retrogradation

INTRODUCTION

WATER ACTIVITY, rather than water content, is the primary factor that determines the lower limit of available water for microbial growth (Beuchat, 1981). Water activity of certain foods must be lowered to a suitable range during storage to minimize microbial spoilage. This can be achieved by adding humectants which bind water and maintain texture. However, relatively few substances such as glycerol, sucrose, glucose, fructose, propylene glycol, and sorbitol, are known to effectively lower a_w for intermediate moisture foods (Bone, 1973; Brockmann, 1970; Chirife et al., 1982). Some such humectants are sweet and fermentable sugars. They might be used by microorganisms as carbon sources, stimulating growth of spoilage microorganisms.

Conventionally branched oligosaccharides (BOS) have been produced by serial reactions of α-amylase, β-amylase, and transglucosidase on starch (Takaku, 1988). The BOS mixture usually included isomaltose, isomaltotriose, and panose *etc.* BOS have desirable humectant properties; low in sweetness, viscosity, and a_w, but high moisture retaining capacity (Takaku, 1988). BOS are also a growth factor for *Bifidobacterium*, a valuable human intestinal flora (Park et al., 1991; Ishibashi and Shimanura, 1993; Oku, 1994; Tomomatsu, 1994). Therefore, BOS might be preferably applied to food processing and preservation to improve physicochemical properties and extend shelf life of certain foods.

We have isolated a gene encoding a maltogenic amylase from *Bacillus licheniformis* (BLMA; Kim et al., 1992) and succeeded in producing a BOS mixture by applying an *E. coli* lysate, which contained both BLMA and a thermostable α-amylase (BLTA), to 15% starch solution (Kim et al., 1994). The liquefaction and saccharification procedures were carried out simultaneously, shortening the process time. The glucose and maltose molecules released from hydrolysis of BLTA were transferred onto the cleavage sites of other sugar molecules by the transglycosylation action of BLMA to form various BOS. However, the yield of BOS from the conventional process was relatively low, and the BOS preparations contained high percentages of glucose and maltose (Takaku, 1988; Lee, 1993). Our objective was to prepare a high BOS mixture by successional yeast fermentation of the BOS mixture. We also investigated the water sorption properties of BOS and evaluated the efficacy of BOS as a humectant in bread.

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MATERIALS & METHODS

Preparation of BOS

Bacillus licheniformis maltogenic amylase (BLMA) was purified from *E. coli* HB101 transformed with the recombinant plasmid containing the BLMA gene as described by Kim et al. (1992). Isopanose was kindly donated by Professor Y. Sakano (Tokyo Noko Univ., Japan). α-Amylase (Termamyl 120L, Type LS) was purchased from Novo Nordisk, Denmark. BOS was enzymatically produced by BLMA from liquefied corn starch. Corn starch suspension (30% w/v) in 50 mM maleate-NaOH buffer (pH 6.8) was liquefied by α-amylase (1.6×10^7 U/g) at 90°C usually for 10 min or until an appropriate degree of hydrolysis (DE) value of the liquefied corn starch syrup (about 22) was obtained. The DE value was measured by dinitrosalicylic acid (DNS) method during the reaction (Kim et al., 1992). The reaction was stopped by autoclaving the hydrolysate at 121°C for 10 min when the DE was ≈20–25. EDTA was added to the final concentration of 5 mM and pH of the reaction mixture was adjusted to 6.8 using 1N NaOH. For synthesis of BOS, 400CU of BLMA/g substrate was added to the solution and the reaction was carried out at 45–50°C for 15 hr. One unit of cyclodextrin hydrolyzing activity (CU) of BLMA was defined as the amount enzyme producing reducing sugar equivalent to one unit change of absorbance at 575 nm. The reaction was stopped by boiling, filtered through a Whatman paper (No. 5), and dried in a freeze drier.

Immobilization of yeast cells

Saccharomyces cerevisiae var. *ellipsoideus* (IFO 1950) was obtained from Korean Culture Center of Microorganisms and used to remove glucose and maltose from the BOS mixture. Sodium alginate (2.5%; w/v) was dissolved in 100 mL of distilled water by heating and then mixed with 100 mL yeast cells cultured in YPD medium (yeast extract 1%, w/v; Bactopeptone 2%, w/v; dextrose 2%, w/v) for 18 hr at 27°C. The mixture was dropped into 0.2M $CaCl_2$ solution using a syringe. The droplets formed beads, (diam 2–3 mm) in the solution. Entrapped yeast cells were washed completely with distilled water and used for production of high BOS mixture.

Preparation of high BOS mixture

The BOS mixture (30%; w/v) was fermented by immobilized *Saccharomyces cerevisiae* var. *ellipsoideus* cells. Fermentation was carried out in a 500 mL fluidized reactor containing the BOS mixture (100 mL)

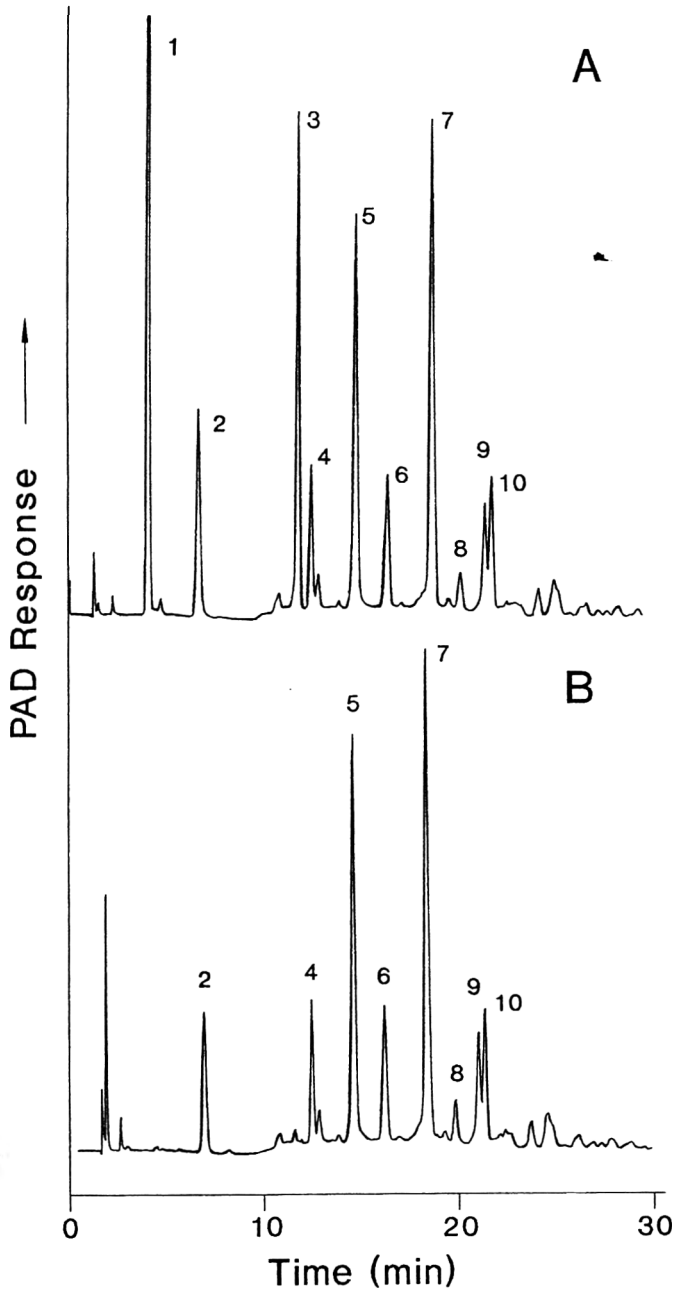

Fig. 1—HPIC analysis of BOS and high BOS mixtures. (A) BOS mixture, (B) high BOS. (1) glucose; (2) isomaltose; (3) maltose; (4) isopanose; (5) panose; (6) maltotriose; (7) branched DP4 molecules; (8) maltotetraose; (9 & 10) branched DP5 molecules.

and immobilized cells (200 mL) at 27–28°C for 2 days with gentle shaking. Upon completion of fermentation, the mixture was filtered and dried.

High performance ion chromatography (HPIC) of BOS and High BOS mixtures

Ion chromatography was carried out using a CarboPac PA1 column (Dionex Bio LC 4500i) and a pulsed amperometric detector (PAD, Dionex). All solvents were degassed by vacuum stirring and filtered through a polyvinylidine difluoride membrane filter with 0.45 μm pores (Gelman Sciences Inc.). Samples were eluted at 1.0 mL/min with linear gradient of 150 mM NaOH (100%–70%) and 150 mM NaOH containing 600 mM sodium acetate (0–30%) for 30 min. The 0.02% (w/v) sample solution (20 μL) was injected into the column for analysis.

Water activity and water content

Water sorption isotherms of BOS mixtures and sucrose were determined by the manometric method measuring equilibrium vapor pressure at different moisture contents. The manometer was constructed in our laboratory and was similar to that reported by Taylor (1961). Moisture of BOS was increased by adding water to 5g of BOS at 5% (w/w) increments. The initial moisture content of the BOS and the high BOS mixtures was 2.8%. Water activity in bread was measured by the same method. A loaf of bread was baked using white pan bread mix I (Jeil Sugar Co.) in a bread machine (Samsung Co., Korea) as described (Kweon et al., 1994). The pan bread mix I contained wheat flour 88.66% (w/w), sugar 3.56% (w/w), shortening 3.53% (w/w), non-fat dried milk 1.76% (w/w), NaCl 1.41% (w/w), yeastfood 0.09% (w/w), vitamin C 0.006% (w/w), and dried yeast 0.99% (w/w). BOS was added at 10% (w/w) or 20% (w/w) of wheat flour where necessary.

Table 1—Sugar composition of BOS and high BOS mixtures

Saccharide	BOS (%)	High BOS (%)
Glucose	22.6	—
Maltose	13.4	0.9
Isomaltose	6.9	7.3
Maltotriose	4.6	7.0
Panose	13.7	21.0
Isopanose	3.8	7.5
Maltotetraose	1.4	2.4
Branched tetraose	18.9	28.6
Maltopentaose	0.8	2.4
Branched pentaose	8.7	14.9
Maltohexaose	0.9	1.6
Branched hexaose	4.3	6.4
> Branched hexaose	trace	trace
Total for branched oligosaccharides	56.3	85.7

Sensory evaluation of BOS

Relative sweetness of BOS and high BOS mixtures was determined by a panel of 8 members. BOS samples were prepared at 10% (w/v). Sucrose solutions at 0.5%, 1%, 2%, and 3% (w/v), designated as A, B, C, and D, respectively, were served as reference solutions along with samples in paper cups randomly coded with 3 digit numbers at room temperature (≈23°C.). Panelists evaluated the intensity of sweetness of samples with respect to sweetness of the references and scored them on a bar with 4-point scale (A, B, C, D). The overall quality of the bread containing BOS was evaluated by a panel of 10 members. Slices of bread containing 0% (w/w), 10% (w/w), or 20% (w/w) BOS were served and panelists evaluated appearance, flavor, moistness, and sweetness of each sample. Scores were marked on a bar with 4-point scale (A, B, C, D). All tests were carried out in triplicate.

Effect of BOS on retrogradation of bread

Three loaves of bread containing 0%, 10%, or 20% of BOS were baked. They were stored at room temperature (≈23°C) in polyethylene bags, and the degree of retrogradation was measured by differential scanning calorimetry (DSC). DSC measurements were performed using DSC 120 (Seiko Co., Japan). Samples (10 mg each) were weighed into aluminum pans (≈20 μL capacity) and heated from 30 to 130°C by 5°C increments/min. In all experiments, a pan containing 10 mg of glycerol was used as a reference (Kweon et al., 1994). The degree of retrogradation of bread was determined by comparing relative areas of endothermic peaks on DSC thermograms. Transition enthalphies (ΔH_t), as calories/g, were calculated from the area under the curve, and recorded as an index of retrogradation. All DSC measurements were carried out in triplicate.

RESULTS & DISCUSSION

Compositions of BOS and high BOS mixtures

Chemical structures of major compounds in the BOS and high BOS mixtures including isomaltose and isopanose, were confirmed by HPIC. (Fig. 1) Good separation was obtained for isomaltose from maltose and isopanose from panose. The branched tetraose and branched pentaose fractions were collected separately and characterized further. HPLC analysis of the branched tetraose fraction treated with pullulanase indicated that >82% of the fraction was degraded to maltose. Therefore, it probably

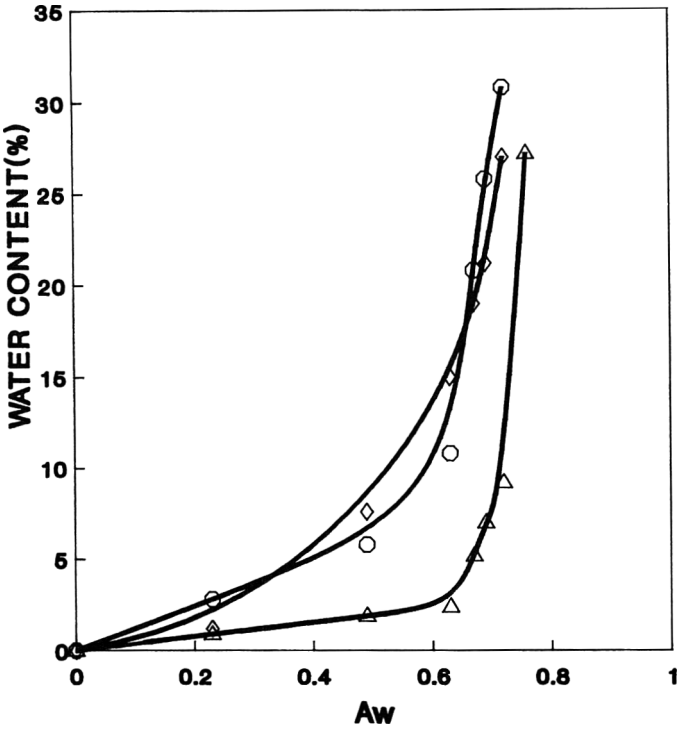

Fig. 2—Water sorption isotherms of BOS, high BOS, and sucrose. Moisture content of samples was increased by adding water at 5% (v/w) increments. ◊ BOS; ○, high BOS; △, sucrose.

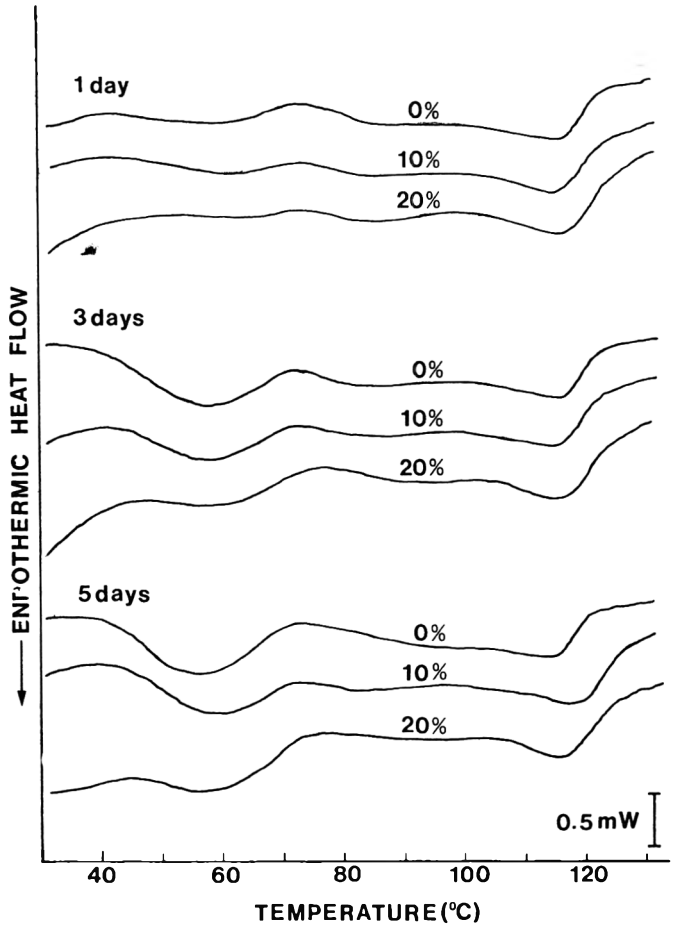

Fig. 3—Retrogradation of bread analyzed by DSC. Breads containing none, 10%, or 20% BOS were analyzed after 2 or 4 days storage at room temperature (≈23°C).

consisted mainly of 6²-O-α-maltosyl-maltose. The branched pentaose fraction was treated with either pullulanase or glucoamylase and analyzed by HPLC and TLC. The fraction was hydrolyzed to maltose and maltotriose by pullulanase, and solely to glucose by glucoamylase. Results suggested that two isomers, 6³-O-α-maltosyl-maltotriose and 6²-O-α-maltotriosyl-maltose, were present in equal amounts.

Peaks of glucose and maltose from the BOS mixture were large in the HPIC chromatogram (Fig. 1A, peaks 1 & 3), but they were very small in the high BOS mixture (Fig. 1B). No difference was observed in peaks of other molecules. This indicated that yeast used glucose and maltose for fermentation, but not BOS. The BOS mixture contained 36% glucose and maltose, while the high BOS mixture contained only 0.9% maltose.

French et al. (1966) tried gradient elution of charcoal columns using aqueous butyl alcohol to separate individual starch oligosaccharides in the range between G2 and G15. However, handling of the charcoal column was not exactly reproducible. Takahashi and Goto (1994) used a continuous rotating annular chromatography (CRAC) with a rotating feed nozzle and product collectors to produce a low calorie sweetener free of mono- and disaccharides by separating mixtures of fructooligosaccharides. They completely separated monosaccharides and oligosaccharides but disaccharides could not be separated from other components. They suggested that separation may be improved by using a longer annular column.

The sugar compositions of the BOS and high BOS mixtures were compared (Table 1). The BOS mixture contained 56.3% BOS including panose, branched tetraoses, and branched pentaoses, etc. BOS was enriched to 85.7% in the high BOS mixture by the yeast fermentation. The BOS and high BOS mixtures were mainly composed of branched molecules with 2, 3, 4, and 5 glucose residues. These mixtures contained more branched tetraose and pentaose than the BOS mixture in current markets (Biomaltooligo-50™), while the commercial product contained more isomaltose (total BOS in Biomaltooligo-50™ was 53%). Generally, glucose in oligosaccharides syrups may cause problems in applying them to food processes. They may stimulate growth of microorganisms and Maillard reactions. The amount of glucose contained in the BOS (22.6%) or high BOS mixture (none) was much lower than that in the mixture reported by Takaku (1988; 45.5%) or by Kuriki et al. (1993; 22.8%). Therefore, our high BOS mixture should cause less browning reactions and produce more favorable properties in foods than other BOS preparations.

Table 2—Changes in relative retrogradation degree of breads containing BOS with storage time[a]

Storage time (days)	Breads: Control ΔH (cal/g)	BOS (10%) ΔH (cal/g)	BOS (20%) ΔH (cal/g)
1	0.0710 ± 0.0042	0.0608 ± 0.010	0.0248[a] ± 0.0056
3	0.213 ± 0.028	0.185 ± 0.0319	0.117 ± 0.018
5	0.263 ± 0.022	0.222 ± 0.0147	0.162 ± 0.042

Source	df	Anova SS	Mean square	F value	Pr > F
Concentration (C)	2	0.0307	0.0154	18.97	0.0001
Days (D)	2	0.1287	0.0644	79.34	0.0001
C*D	4	0.0029	0.0007	0.89	0.4893

[a] Mean values of three observations ± Standard deviation

Effect of BOS on a_w and moisture

Water sorption isotherms of the two BOS mixtures and sucrose were compared (Fig. 2). The isotherm levels of BOS were higher than those of sucrose at a_w, 0.1–0.8. The moisture content of sucrose was about 9.2% at a_w 0.72, while that of BOS and high BOS was 27% and 30.8%, respectively. Sucrose had a_w of

0.75 when moisture was 10%. It corresponded to a_w of 0.6 in the BOS mixture with the same moisture content. Thus a_w decreased at a constant moisture content in the whole range of water activities. These properties would be of benefit to control microbial contamination in intermediate moisture foods (Labuza et al., 1972).

Water activity of bread decreased from 0.87 to 0.82 or to 0.78 when it contained 10% or 20% BOS, respectively. Thus, BOS could decrease a_w of certain foods effectively, thereby preventing microbial spoilage. Although several mono- and disaccharides, especially sucrose, can lower a_w, they may stimulate microbial growth and/or Maillard browning reaction since they are fermentable and reducing sugars. However, the BOS mixture was neither fermentable nor sweet, so that it may help improve physicochemical properties when added as a humectant. The BOS mixture acted as a water molecule plasticizer in the bread. The bread containing BOS (20%) had more glossy crust, more uniform and compact texture than the control bread.

Sensory evaluation of BOS and high BOS mixtures

Relative sweetness of 10% (w/v) BOS and 10% (w/v) high BOS solutions were ≈1.75% and 1.1% of 10% (w/v) sucrose sweetness, repectively. Sweetness of sugars varies with constitution, configuration, physical form of sugars, and degree of polymerization of sugar components (Shallenberger and Acree, 1967). Lindley and Shallenberger (1976) reported that sweetness of various sugars was dependent on degree of intramolecular hydrogen bonds within the sugar molecules. They also suggested that the ring oxygen atom might act as an electronegative center causing increases in sweetness and reductions in bitterness. Thus, glucose and maltose would likely be more sweet than BOS with degree of polymerization ≥3. The sweet taste of the BOS mixture could be reduced by removing glucose and maltose. Low sweetness of BOS and high BOS mixtures would also probably reduce dental caries when added as food humectants (Hamada et al., 1984).

Breads containing BOS had more uniform and compact texture than the control bread, but they scored almost the same for appearance and flavor. The panelists scored the bread with 20% (w/w) BOS highest and the control bread lowest in moistness. The control bread and that with 10% (w/w) BOS were evaluated as less sweet at the same degree as the bread with 20% (w/w) BOS. The evaluation of moistness and sweetness was analyzed with 95% and 99% confidence, respectively. The bread sample containing 10% (w/w) BOS was preferred to other breads in overall quality.

Effect of BOS on retrogradation of bread

Degrees of retrogradation of breads were measured by comparing areas of endothermic peaks in DSC thermograms (Fig. 3). The first endothermic peak appeared at 60°C to 85°C. Enthalphy of bread (ΔH) used as the index of retrogradation was estimated from the area of the peak (Table 2). Enthalphies of bread without BOS were 0.213 cal/g and 0.263 cal/g after 3 days and 5 days storage at room temperature, respectively. Enthalphies of bread containing 10% BOS were 0.185 cal/g after 3 days and 0.222 cal/g after 5 days storage. Enthalphies of bread containing 20% BOS were the least; 0.117 cal/g and 0.162 cal/g after 3 and 5 days of storage, respectively. Therefore, starch retrogradation was retarded probably due to steric hinderance and moisture retention by BOS. The effect of BOS on retarding retrogradation of bread seemed to increase as concentration of BOS in the bread increased. BOS would be a good humectant for foods and the properties of BOS would be improved by processing it further to high BOS via yeast fermentation to reduce sweetness.

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Tribological Shear Conversion of Starch

SHAW S. WANG and XIAOGE ZHENG

ABSTRACT

Tribological (powdery friction) conversion (cooking) of starch as followed by DSC measurements was studied in a capillary rheometer. The crumbly extrudates with <20% conversion were analyzed to obtain a kinetic model and its parameter values suitable to describe the phenomena of tribological conversion of starch. Such conversion of starch followed zeroth order kinetics. An Arrhenius type activation energy plot described the shear energy initiated conversion well. The shear activation energy of starch conversions due to tribological shearing of powders was lower than that found for overall (tribological plus rheological) conversions. These findings can help develop more accurate and realistic models for starch conversion and similar processes and help elucidate their mechanisms.

Key Words: Powdery starch, Shear energy, Extrusion, Tribological Conversion

INTRODUCTION

COOKING OF STARCH in excess water leads to its gelatinization. Wang et al., (1991) reported that regardless of sources of starch, the amount of water needed for complete gelatinization was 14 molecules for every anhydro-glucose unit of starch. That is essentially a 61% water and 39% starch mixture. So, for starch-water mixture with <61% water, the amount of water in the mixture is not in excess stoichiometrically for formation of gelatinized starch. Under limiting water contents, if there is enough energy available to such mixtures, the fraction of starch that is not gelatinized could be melted (Wang et al., 1991). Here, "melting" is the change indicated in the thermogram found in DSC (Differential Scanning Calorimetry) scans. Under such limiting water (<61%) conditions, DSC scans have shown two endothermic peaks. The first corresponds to gelatinization, and the second to "melting." Melting requires enthalpy, ΔH, which is measurable in the DSC peak. (Qu and Wang, 1994). For example, biscuits and many extruded products are cooked under limited water contents and such products contain melted in addition to gelatinized starch. Conventionally thermal energy is the sole source in baking and boiling, but in extrusion, shear energy is also important. The question is whether shear energy can, like thermal energy cause part of the "melting" of starch. Through studies of starch extrusion at temperatures <40°C, Wang et al. (1992) and Zheng and Wang (1994) concluded that shear energy alone, could cook starch according to DSC (Differential Scanning Calorimetry) measurements.

Kinetic behavior of starch cooking has been mostly first order for systems with excess water (Suzuki et al., 1976; Kubota et al., 1979; Shiotsubo, 1983; Lund and Wirakartakusumah, 1984), and zeroth order for those with limited water (Wang et al., 1989; Wang, 1993; Zheng and Wang, 1994). Extrusion conversion of starchy materials has also been reported to follow first or second order kinetics (Diosady et al., 1985; Cai and Diosady, 1993). These are process kinetics (as opposed to intrinsic kinetics) resulting from experiments with varying temperature and/or shear forces in extrusion processes.

Intrinsic rate constants are functions of temperature and shear energy inputs. Ideally temperature and shear energy inputs need to be held constant in experiments designed to find intrinsic rate constants. Extrusion processes are generally done with moisture <40%. If the extruded starch is fully cooked the majority of it is melted with <26% being gelatinized according to the stoichiometric relationship. Melting is a zeroth order process. This is probably the key reason that the overall process of extrusion cooking of starch is zeroth order.

Another interesting aspect of extrusion is that, in general, more than half the extruder length contains some material in powdery state (Yam et al., 1994, Wang et al., 1994). Study of the interactions and frictions among powders and other surfaces is called tribology. We have studied overall extrusion cooking of starch in single and twin screw extruders and in a capillary rheometer. In those studies, firstly, powdery starch particles interacted among themselves and with the surface of the machinery through tribological friction in the metering and beginning of the compression zones. There must be a certain degree of conversion (cooking) of starch at that stage to make the originally nonaggregating powder "sticky." The tribologically developed "stickiness" causes the sol-gel transition and starch particles then aggregate into a rheological mass (Wang et al., 1994). Secondly, in the compression zone that is close to the die, further conversion (cooking) of starch occurs in the material that is in rheological state. Note that we equate starch conversions to degrees of cooking because in evaluations of DSC data, enthalpy (thermal energy) was used to calculate degrees of conversions. (Wang et al., 1989).

The kinetic studies we have reported have been overall conversions, including those in both tribological and rheological zones of extruders (Wang et al., 1989, 1992; Zheng and Wang, 1994). We were interested in studying tribological conversion of starch separately from rheological conversion to see if it were different from the overall studies. In order to accomplish this we had to design experiments that would produce extrudates still in tribological but not in rheological state. This also meant that we had to produce extrudates that did not hold their shapes, for example, crumbly noodles. Such products are relatively low in percent conversion (degree of cooking). From model studies, Wang et al. (1994) reported that for waxy corn starch (35% moisture), 36% conversion of the starch was needed for a hydraulically pressed cake to hold its integral shape when subjected to a specified mechanical vibration force. Our objective was to investigate the kinetics of this relatively low degree of conversion. For conversion of starch in the powdery form, thermal energy would probably be less effective than shear energy due to the resistance of heat transfer and the fact that starch molecules with molecular weights much higher than one million are very slow in acquiring kinetic energy from thermal activation. In a capillary rheometer or an extruder, powders are compressed, so the probability of particle friction would be increased which would enhance the tribological effects. We designed experiments to study the tribological conversion of waxy corn starch with moisture content commonly used in food extrusion.

MATERIALS & METHODS

THE WAXY CORN STARCH (amylopectin content 97%) reported in previously publications was used. Moisture content in the raw sample determined and calculated amounts of water were added to adjust sa

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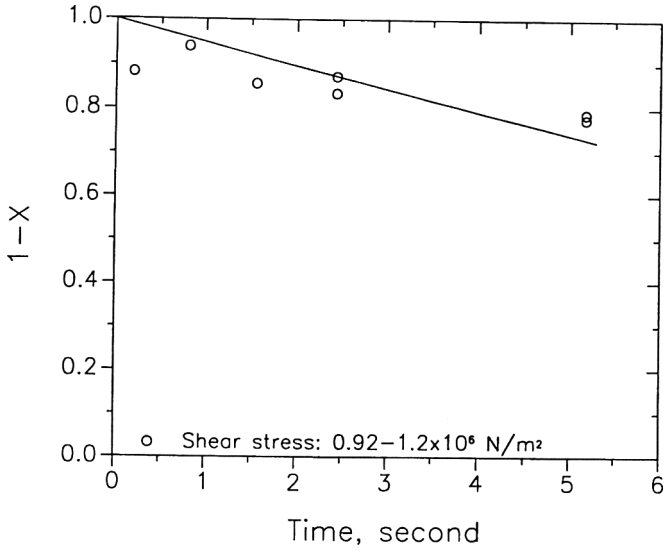

Fig. 1—Tribological kinetics of powdery waxy corn starch with 35% water extruded in a capillary rheometer. The fraction of unconverted starch (1-X) was related to reaction time. Energy input was held at 40°C and a shear stress from 0.9 × 10^6 to 1.2 × 10^6 N/m^2, as nearly constant shear stress as possible.

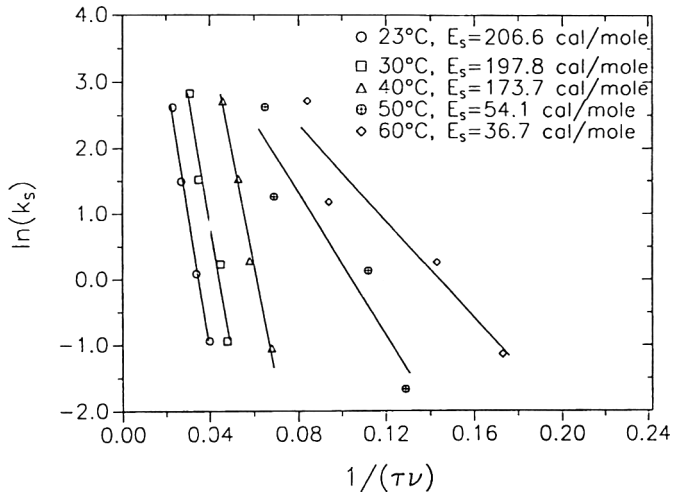

Fig. 2—Activation energy plot for tribological conversion of waxy corn starch with 35% water in a capillary rheometer. (Refer to Eq. (7) in the text) k_s is the shear initiated rate constant, in min^{-1}, $\tau\upsilon$, the product of shear stress and molar volume, (calories/mole).

ples to required water contents. We used a Perkin Elmer DSC-4 to measure degree of conversion (Wang et al., 1989).

A capillary rheometer (Model 3211, Instron Co. MA) was used as an extruder to study low conversion under various shear forces. Experiments were designed to extrude raw starch to partially converted (cooked) powdery starch by selecting a capillary cylinder with relatively large diameter (0.21 cm) and short length (2.1 cm). Different load forces (ranging from 900 to 6450 N) were used at constant temperature to study effects of shear stress on degree of conversion. Effects of temperature (ranging from 23°C to 60°C) were also studied. Different plunger speeds (0.25, 0.76, 2.54 and 7.62 cm/min) were used to obtain extrudates with different reaction time. Data collection included calculations of degrees of conversion from DSC data, of reaction time from Eq. (1) and (2), of rate constants from each set of constant temperature and shear stress experiments, and of shear activation energy and the pre-exponential coefficients from linear correlations of natural log of rate constants vs the product of shear stress and molar volume of the anhydro-glucose unit in the starch-water mixture (Eq. 7). An average shear stress used in each experiment was calculated from Eq. (3) and (4), and molar volume of glucose from Eq. (5).

$$t = \frac{l_c}{V} \tag{1}$$

$$V = \frac{V_{xh}}{60} \cdot \left(\frac{d_b}{d_c}\right)^2 \tag{2}$$

$$\tau_w = \frac{F}{4\,A_p \cdot (l_c/d_c)} \tag{3}$$

$$\tau = \frac{2}{3} \cdot \tau_w \qquad \text{(Goodman and Bestul, 1955)} \tag{4}$$

$$\upsilon = \frac{16200}{A_s \cdot \rho_s + (100 - A_s) \cdot \rho_m} \tag{5}$$

$$(1 - X) = -k_s t \tag{6}$$

where t = reaction time, sec.; l_c = length of capillary, cm; V_{xh} = plunger speed, cm/min; d_b = barrel diameter of capillary cm; d_c = capillary diameter, cm; V = mean velocity of material going through the capillary, cm/sec; τ_w = shear stress at the capillary wall, N/m^2; F = load force, N, A_p = cross section area of the rheometer barrel, cm^2; τ = average shear stress, N/m^2; υ = molar volume of glucose in the starch-water mixture, cm^3/mole; A_s = weight percent (wet base) of starch; ρ_s = specific gravity of dry starch, g/cm^3; ρ_m = specific gravity of water, g/cm^3; X = degree of conversion, dimensionless; and k_s = shear initiated reaction rate constant, min^{-1}.

Table 1—Kinetic parameters of shear induced tribological (powdery friction) and overall conversion (including both tribological and rheological) of waxy corn starch with 35% moisture at relatively low temperatures in a capillary rheometer[a]

Tribological kinetics			Overall kinetics		
Temp. °C	E_s (cal/mole)	k_{So} (min^{-1})	Temp. °C	E_s (cal/mole)	k_{So} (min^{-1})
23	207	1.37×10^3	21	288	6.63×10^7
30	198	6.44×10^3	25	245	1.41×10^4
40	174	4.18×10^4	35	191	3.42×10^4
50	54	2.84×10^2	40	100	5.46×10^3
60	37	1.98×10^2	50	65	1.08×10^2

[a] Tribological kinetic parameters were obtained from experiments with percent conversions < 20%, and that of overall kinetics from experiments with percent conversions > 40% (Zheng and Wang, 1994).

RESULTS & DISCUSSION

WANG ET AL. (1994) reported that for waxy corn starch with 35% moisture content, the powdery starch would be sticky enough to pass the sol-gel transition and form an aggregated rheological mass if percent conversion of the starch exceeded 36%. That amount of conversion was achieved by tribological shearing (friction) of granules in the extruder if the moisture content in the starch feed was 35%. Yam et al. (1994) analyzed materials along the screw channel after "dead-stop" of a steady state twin screw extrusion of corn meal with 20% moisture. They reported that up to conversion between 20 and 32% the material was obviously in a powdery state. In order to make sure that we were only converting starch when it was still in a powdery state, we only used data of samples that had 20% or lower conversions of starch to obtain kinetic parameters in this study. Temperature was maintained at 23, 30, 40, 50 and 60°C for each set of plunger speeds (0.25, 0.76, 2.54 and 7.62 cm/min). The fitting of zeroth order kinetics to the shear induced tribological conversions of starch at 40°C (Fig. 1) and a practically constant shear stress ranged from 0.92 × 10^6 to 1.2 × 10^6 N/m^2. Note that the reaction rate constant, k_s, is a function of energy input. In each kinetic experiment designed to calculate the rate constant both thermal and shear energies were kept as nearly constant as possible. Due to configurational limitations, an absolute constant shear stress is difficult to maintain in the capillary rheometer.

A fairly good linear correlation (Fig. 2) was found between ln k_s, the natural log of shear initiated rate constant, and the reciprocal of $\tau\upsilon$, the product of shear stress and molar volume of anhydro-glucose unit in the starch-water mixture. This suggested that the following equation was suitable for describing the kinetic behavior of starch conversion (cooking) caused by shear forces applied.

$$k_s = k_{s,o} \cdot e^{-E_s/\tau\upsilon} \quad (7)$$

We used a zeroth order model to calculate the rate constant, k_s (Fig. 1) Generally, a zeroth order rate constant has a dimension of concentration divided by time, e.g. M/L/min. Since we used percent conversion X, (a dimnensionless parameter) to follow the reaction kinetics, the dimension of k_s is reciprocal of time. Equation (7) has also been shown to describe overall starch extrusion kinetics, including both powdery (tribological) and rheological conversion, well (Wang et al., 1992). The form of Eq. (7) is very similar to that of the Arrhenius equation, which relates natural log of reaction rates to reciprocal of temperature and gas constant, R, with thermal activation energy as the slope of the correlation. The product of RT is a measure of the kinetic, potential and other energies of gas molecules in the reaction system at that temperature. Here the shear stress initiated reaction rates were related to a similar shear energy parameter, $\tau\upsilon$, the product of shear stress and molar volume. The products of RT and $\tau\upsilon$ have the same physical meaning, and their dimensions are both calories/mole or joules/mole. (The dimension of shear stress (τ) is dynes/cm^2 and that of molal volume (υ) is cm^3/mole. So the product of $\tau\upsilon$ has a dimension of dyne-cm/mole which can be adjusted to joules/mole, or calories/mole.) The shear activation energy, E_s, is a function of temperature. In the range we studied (23°C to 60°C), the higher the temperature the lower the E_s. The kinetic parameters, E_s and $k_{s,o}$, the pre-exponential coefficient, of tribological conversion and overall conversion (tribological plus rheological conversion) were compared (Table 1). The shear activation energies of tribological kinetics were consistantly lower than those of overall kinetics when both were studied in comparable temperature ranges. This suggested that tribological shear was more efficient than rheological shear in converting starch. As for $k_{s,o}$, the pre-exponential coefficient, the values are comparable at temperatures higher than 40°C. However, the values for tribological conversion were considerably smaller than those for overall conversion when the temperature dropped to 23°C. From the practical point of view, these kinetic equations and parameters are useful in modeling extrusion processes. From the scientific point of view, it is interesting to know that shear energy initiated tribological changes do cause conversion (cooking) of starch as revealed by DSC measurements.

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Enhanced Sweetness in Sweetener-NaCl-Gum Systems

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ABSTRACT

Enhancement of sweetness in aqueous gum (0.03%, w/v) sweetener systems by added NaCl (0.05%, w/v) was evaluated by a sensory panel. ^{23}Na NMR spectroscopy was used to determine Na^+ binding and its relationship to sweetness elicited by glucose, lactose, maltose, sucrose and aspartame. Sweetness intensity differed due to gum ($p = 0.0001$) and sweetener ($p = 0.0001$), but was not affected by NaCl ($p = 0.0774$). Sweetness increased with added NaCl in xanthan, guar and locust bean gum solutions. However, sweetness decreased in κ-carrageenan systems possibly due to endogenous cation (Ca^{2+}, K^+ and Na^+) content, which influences Na^+ mobility. The sweetest systems containing lactose and/or xanthan, showed the greatest enhancement by NaCl.

Key Words: sweetness, NaCl, gums, sugars, aspartame

INTRODUCTION

LOW LEVELS OF NaCl have been hypothesized to enhance sweet taste (Fabian and Blum, 1943; Hahn and Ulbrich, 1948; Anderson, 1950). However, the general effect of mixing 2 taste components is usually a reduction of both taste qualities (Pangborn, 1974; Bartoshuk, 1975). Bartoshuk (1975) indicated that the taste of a mixture was the result of individual components, and although the general effect was masking, enhancement could occur if both components contributed to the same taste. NaCl exhibits a slightly sweet taste, which may explain the enhanced sweetness of dilute NaCl plus sucrose solutions (Bartoshuk, 1975). Similarly, Kroeze (1982) reported that NaCl had a "side taste" that was sweet. The side taste was defined as a taste sensation qualitatively different and lower in intensity than the main taste of a stimulus.

Early studies of sucrose/NaCl solutions showed enhanced sweet taste when low levels of both tastants were used. Fabian and Blum (1943) found that near or subthreshold levels of NaCl (0.025M) in aqueous solutions increased the sweetness of suprathreshold concentrations of mono- and disaccharides, while subthreshold sweetener levels reduced saltiness. Hahn and Ulbrich (1948) reported that sweet thresholds of sucrose decreased with added NaCl, while salt thresholds were not affected by sucrose. Anderson (1950) observed that at threshold levels of NaCl and sucrose, NaCl enhanced sweetness, while sucrose slightly decreased saltiness. Kamen et al. (1961) noted that NaCl enhanced sweetness of 0.5% sucrose.

Suprathreshold levels of NaCl have also been related to enhanced sweetness in sucrose solutions. Beebe-Center et al. (1959) found that sweetness of 1% sucrose was slightly enhanced by NaCl up to 3%, and sweetness of 10% sucrose was enhanced by addition of 0.3% NaCl. Pangborn (1962) reported that sweetness of 0.75% and 2.25% sucrose solutions was enhanced by 0.36% NaCl. According to Pangborn (1987), contradictory findings on the effects of NaCl on sweetness might have been due to different sensory testing methods. There appears to be a relationship between the two tastes but inconsistent results indicate the need for further study.

An amiloride-sensitive pathway is believed to be involved in both salty and sweet tastes (Schiffman et al., 1983). Simon (1990) proposed a "Direct Activation Model" for sweet taste transduction that included an amiloride-sensitive pathway that allows the flow of the Na^+ ion following the binding of a sweetener. The Na^+ ion starts a "transduction cascade" (Lancet and Ben-Arie, 1990), which leads to depolarization and enhanced sweet taste. Thus, in the presence of a sweetener, low levels of NaCl could participate in sweet taste transduction through the amiloride-sensitive pathway.

Earlier work in our laboratory showed that ionic gums (xanthan and κ-carrageenan) depressed saltiness perception at concentrations near the saltiness recognition threshold compared to non-ionic (guar and locust bean) gums when all gums were at, 0.1% and 0.3% levels. Viscosity and pH were not influential factors (Rosett et al., 1994, 1995). ^{23}Na nuclear magnetic resonance (NMR) spectroscopy and sensory studies of NaCl/gum solutions suggested that ionic gums "bound" Na^+ when it was at low levels, making it less mobile and unavailable for perception (Rosett et al., 1994, 1995). They also indicated that inherent cations, such as potassium and calcium, may be preferentially "bound" to ionic gums, releasing the Na^+ for salt perception.

Little has been reported about relationships of NaCl with other sweeteners such as glucose, lactose, maltose, and aspartame. Since ionic gums bind Na^+, and make it unavailable for salt taste transduction, we hypothesized that it would also be unavailable to contribute to sweet taste. If Na^+ is bound to an ionic food ingredient such as a gum, then Na^+ could not start the "transduction cascade" in the "Direct Activation Model." Thus, ionic gum/sweetener/NaCl solutions should not be as sweet as nonionic gum/sweetener/NaCl solutions. We have demonstrated the ionic binding of Na^+ and corresponding saltiness suppression at near saltiness threshold levels (Rosett et al., 1994, 1995). Additionally, earlier studies (Fabian and Blum, 1943; Anderson, 1950; Kamen et al., 1961) had reported sweet taste enhancement at near threshold levels of sucrose and NaCl. Our objectives were to investigate relationships between sweet and salty tastes in sweetener, NaCl, ionic/non-ionic gum solutions at near sweet and salt threshold levels where a shift from enhancing to masking of tastes may occur (Kamen et al., 1961). This study could provide indirect support for the existence of a sweetener induced pathway for Na^+ that leads to enhancement of sweet taste.

MATERIALS & METHODS

Sweetener solutions

Two ionic hydrocolloids, xanthan and κ-carrageenan, were donated by Kelco, Division of Merck & Co., Inc. (Chicago, IL), and FMC Corporation (Rockland, Maryland), respectively. Two non-ionic gums, locust bean and guar, were gifts from TIC Gums, Inc. (Belcamp, MD). Sucrose (certified A.C.S.) and deionized ultrafiltered water were purchased from Fisher Chemical (Itasca, IL). NaCl and glucose (dextrose anhydrous) were procured from EM Science (Gibbstown, NJ). D-maltose monohydrate (90% pure) and aspartame (L-aspartyl-L-phenylalanine methyl ester, 96% pure) were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Lactose monohydrate powder was supplied by J.T. Baker, Inc. (Phillipsburg, NJ).

Gum concentration was constant at 0.3% (w/v). Based on salty thresholds found in our pilot study with our subjects, 0.05% (w/v) NaCl was used. Since sweet threshold levels of sucrose/NaCl/gum solutions were 0.33–0.49% in the pilot study, 0.5% sucrose was selected. Our goal was to use sweetness levels near the recognition threshold, so we calculated estimated threshold levels at isosweet equivalency to 0.5% sucrose for other sweeteners, based on published data: 0.0025% aspartame, 0.07%

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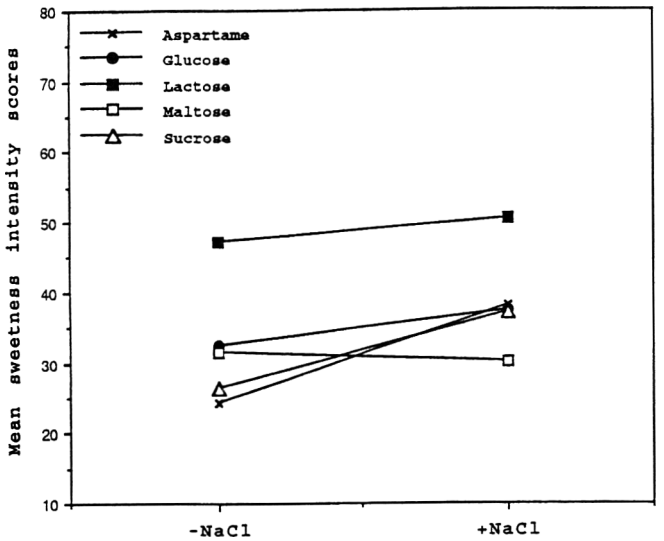

Fig. 1—Mean sweetness intensity scores for sweeteners in aqueous solutions with (−NaCl) and without (−NaCl) NaCl. (n = 39: 13 subjects × 3 reps)

glucose, 3.13% lactose, and 1.52% maltose (Ockerman, 1975; Tver and Russell, 1989).

Deionized ultrafiltered water (500 mL) was preheated in beakers in a water bath at 62.5 ± 2.5°C for 30 min. Gum was added while stirring vigorously. This was immediately followed by addition of NaCl and/or sweetener. Solutions were continuously stirred until NaCl and sweetener dispersed. Bath temperature was increased to 77.5 ± 2.5°C, and beakers were shaken at 2 rpm. Solutions were stirred every 15 min and removed after all additives had been dissolved. Heating times were: 45 min, κ-carrageenan; 60 min, xanthan; 120 min, guar and locust bean. Samples were cooled and held at room temperature (≈22°C) until evaluated by the sensory panel within 36 hr.

Physicochemical measurements

pH of solutions was measured using a Fisher Accumet pH Meter, Model 830 (Fisher Scientific Company, Pittsburgh, PA). A Brookfield Viscometer, Model VTDV-I with UL Adaptor (Stoughton, MA) was used to measure viscosities at speeds 100 and 50. Viscosity values (cps) were obtained by multiplying shear rates by shear stress factors (supplied by manufacturer). Viscosity values <6.4 were indicated to be inaccurate by the manufacturer, and were eliminated from analysis. Xanthan gum solution viscosity was not measured due to its viscoelastic behavior, and viscosity of aqueous solutions with sweeteners and NaCl were <6.4. Viscosity and pH readings were measured at 22°C. Triplicate measurements were done and analyzed by ANOVA for effects of gum type and added NaCl (SAS Institute, Inc., 1990).

Cation contents of the four gums and five sweeteners were analyzed using inductively coupled argon plasma spectroscopy, similar to the method used by Rosett et al. (1994, 1995). Concentration of metals was determined with a Thermo Jarrell-Ash MARK III Model 1100 vacuum direct reader (Thermo Jarrell-Ash, Franklin, MA).

^{23}Na NMR spectroscopy was used to measure the mobility of the Na^+ ion in solutions containing added NaCl, by the method reported by Rosett et al. (1994, 1995). Transverse relaxation rates (R_2, sec^{-1}) were calculated from the line width at half-height ($\Delta\upsilon_{1/2}$) $R_2 = \pi\Delta\upsilon_{1/2}$. Triplicates were analyzed. Effects of gum type were determined using ANOVA (SAS Institute, Inc., 1990).

Moisture contents were determined for sucrose/NaCl/gum solutions to ensure that different heating times did not affect solids content. Samples (≈10g each) were placed in a small glass container. Most of the water was evaporated by placing the containers over a water bath and drying was completed in a vacuum oven overnight at 70°C, 76.2 mm Hg. Triplicates were analyzed. Effects of gum type and ionic/non-ionic gum nature were determined using ANOVA (SAS Institute, Inc., 1990).

Sensory evaluation procedures

The panel consisted of twelve females and one male Univ. of Illinois students or staff members. Subjects completed a series of paired comparison tests using solutions similar to those in intensity rating tests. They were also familiarized with attribute rating procedures during training sessions. For all testing, subjects were seated in individual booths with incandescent lighting, in a constant environment at room temperature (22°C). Samples were served at room temperature in ≈30 mL disposable plastic cups identified by three-digit random numbers. Subjects rinsed with deionized ultrafiltered water before tasting each sample, and expectorated both sample and rinse water.

Saltiness thresholds in NaCl/aqueous solutions and sweet thresholds in sucrose/aqueous solutions were determined using a two-alternative forced choice method (Meilgaard et al., 1992). Concentrations (w/v) of 0, 0.02, 0.04, 0.06, 0.08, 0.10, 0.13, 0.15, 0.18 and 0.20% NaCl (Jellinek, 1985), and 0, 0.05 to 0.85% (increasing by 0.05% increments) sucrose in ultrafiltered deionized water were paired with ultrafiltered deionized water. Subjects were asked to taste each pair of samples and identify the saltier or sweeter sample. Subjects were instructed not to swallow samples, to rinse with water before each sample pair, and to wait at least 15 sec between samples and pairs. Ten tests for both salty and sweet thresholds were performed. To minimize panelist fatigue, four sets of six pairs were presented per session.

Recognition thresholds (50% correct identifications) were determined for each subject, in order to identify any who were not sensitive to salt or sweet taste at the concentrations used. All subjects were retained on the panel because individual thresholds were below 0.10g/100 mL NaCl for saltiness and 0.3g/100 mL sucrose for sweetness reported in Jellinek (1985).

For attribute ratings, different sample combinations were prepared for each sweetener. The ten combinations consisted of the sweetener alone (1), sweetener plus NaCl (1), sweetener plus each of the gums (4), or sweetener plus each of the gums plus NaCl (4). Subjects evaluated ten samples at a time, 20/session, for six attributes: sweetness, saltiness, bitterness, metallic taste, sourness, and thickness. Samples were presented randomly and coded by three-digit random numbers. Each sample was evaluated 3 times. A 150 mm unstructured scale was used, with four anchor words (e.g., not sweet, slightly sweet, somewhat sweet, very sweet) positioned equidistantly under the line from least (left end) to most (right end) intense, for the attribute. Panelists indicated their perception of the attribute by marking the intensity with a slash on the scale provided.

Statistical analysis

Results of physicochemical tests were analyzed using SAS (SAS Institute, Inc., 1990). When treatment effects were significant ($\alpha = 0.05$), Tukey's Honestly Significant Difference (HSD) test was used, controlling for family-wise error rate. Sensory data were analyzed statistically using ANOVA and Tukey's HSD test to determine effects of added NaCl, gum type and property, and sweetener type on sweetness.

RESULTS & DISCUSSION

pH and viscosity

pH values ranged from 3.75 to 7.05, similar to those of 4.1 to 6.2 found in our previous study with the same 4 gums (Rosett et al., 1994). A significant increase in pH occurred when NaCl was added for aspartame, lactose, maltose, and sucrose in gum solutions. Gum type had a significant effect on pH of solutions for all sweeteners tested. Locust bean solutions had a lower mean pH range (4.66–4.89) than κ-carrageenan (6.68–6.82) and xanthan (5.65–6.03). Rosett et al. (1994) reported greater average pH values for κ-carrageenan. The NaCl-gum interaction was significant for pH of aspartame, lactose, maltose, and sucrose solutions. The pH of xanthan solutions with and without NaCl did not change, but all other gums had higher pH with NaCl.

Mean viscosity values (in centipoise) for sweetener solutions with guar and locust bean were not affected by added NaCl. No NaCl-gum interaction occurred, consistent with previous findings (Rosett et al., 1994). Gum type had a significant effect on the viscosities as measured at speeds 100 and 50 for aspartame, glucose, lactose, and maltose, with guar consistently more viscous than locust bean. Since the effects of added NaCl and NaCl-gum interaction were not significant, viscosity was probably not a factor in sweetness determination within a gum type.

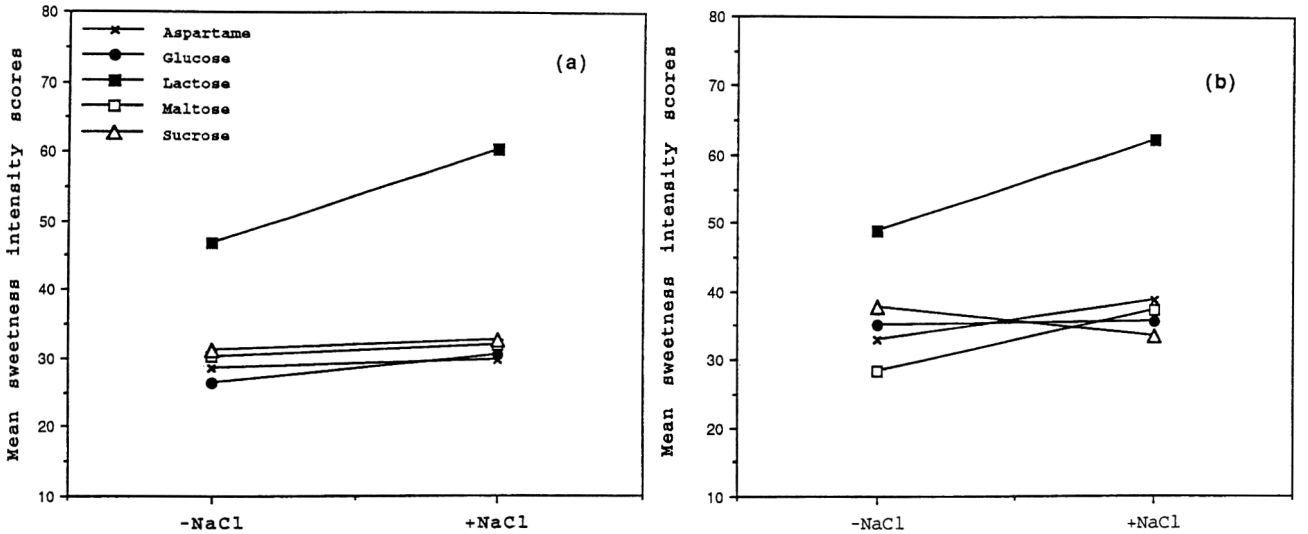

Fig. 2—Mean sweetness intensity scores for sweeteners in (a) guar and (b) locust bean solutions with (+NaCl) and without (−NaCl) NaCl. (n = 39: 13 subjects × 3 reps)

Cation content and Na^+ mobility

The total Na^+ present in a solution needs to be considered, since both inherent and added Na^+ may contribute to saltiness, and perhaps to enhanced sweetness (Ayya, 1988; Rosett et al., 1994, 1995). Solutions with added Na^+ contained 0.05% NaCl, equivalent to 198 ppm added Na^+. Mineral analysis showed that all sweeteners contained <1.900 ppm Na^+ (except aspartame: 57.830 ppm), and <0.1326 ppm Ca^{2+} and 2.195 ppm K^+. Guar and locust bean gums contained <2 ppm Na^+. κ-carrageenan and xanthan gums contained nearly equal amounts of endogenous Na^+ (24.759 ppm and 28.842 ppm, respectively). Shirley (1991) reported similar Na^+ levels in κ-carrageenan and xanthan, but Wu (1993) reported that xanthan contained more than twice the Na^+ as κ-carrageenan, indicating that different lots vary in cation content. κ-carrageenan contained much more Ca^{2+} (83.586 ppm) than xanthan (4.332 ppm), and the non-ionic gums contained much less Ca^{2+} and K^+ than the ionic gums.

^{23}Na NMR are used to determine Na^+ ion mobility in solution. Higher R_2 (sec^{-1}) values indicate less mobility on average; i.e. the mobility of the Na^+ ion is slowed when it is associated with the gum. This "binding" would be influenced by the fast exchange of the ions in the system. R_2 values can be affected by pH, viscosity, and Na^+ level (Ayya, 1988). However, we found that added NaCl did not affect viscosity. Previous research showed that R_2 values were not pH dependent in 0.1% xanthan solutions with 0.1% added NaCl in a pH range of 2.05–11.25 (Shirley, 1991). Therefore, R_2 values are mainly a function of the state and amount of Na^+.

Gum type significantly affected R_2 values; those for xanthan were higher than for κ-carrageenan, locust bean, or guar, suggesting that the Na^+ was less mobile in xanthan solutions than in the other gum solutions. The Na^+ ion, therefore, must have interacted with the available charged groups on the xanthan molecule. Also, the low average R_2 values for κ-carrageenan suggested that it behaved more like the two non-ionic gums than like xanthan (the other ionic gum). Previous research showed that Ca^{2+} and K^+ were bound to xanthan preferentially over Na^+ (Rosett et al., 1995). High levels of Ca^{2+} and K^+ might preferentially occupy available binding sites, leaving Na^+ more mobile. Thus, Na^+ would be unable to interact with the negatively charged groups on κ-carrageenan, and would behave like a non-ionic, non-interactive gum. Ionic/non-ionic nature of the gum had a significant effect on R_2 values, due to the large values for xanthan.

Percent moisture for the four different gum systems with 0.5% sucrose and 0.05% NaCl showed that although solutions were heated for varying times, no significant differences occurred in solute concentrations between ionic and nonionic gum systems. NMR R_2 values, therefore, were not affected by variations in NaCl concentrations. The length of heating time was also not considered a factor in the sensory study, since concentrations of solutes did not vary.

Factors affecting overall sweetness

Sweetness intensity values differed due to gum type ($p = 0.0001$) and gum-NaCl interaction ($p = 0.0006$), as well as sweetener type ($p = 0.0001$). The overall effect of added NaCl on sweetness was not significant ($p = 0.0774$), nor was the sweetener-NaCl interaction ($p = 0.8072$). The significant NaCl-gum interaction was due to decreased sweetness with added NaCl in κ-carrageenan solutions, compared to increased sweetness in other solutions.

Average sweetness intensity values ± standard error of the mean (n = 390) for κ-carrageenan solutions (26.0 ± 1.38) were significantly lower than for guar (34.8 ± 1.51), locust bean (39.0 ± 1.69), xanthan (47.2 ± 1.93) and aqueous (35.5 ± 1.57) solutions. Xanthan solutions were significantly sweeter than guar, locust bean, κ-carrageenan, and water solutions. We had also noted that sucrose/xanthan solutions were sweeter than other gum solutions with the same amount of sugar in our pilot study. Differences in sweetness among gums may have been due to taste properties unique to each gum, and could also be influenced by the level of inherent cations.

Mean sweetness intensity (n = 390) was significantly lower in aspartame solutions (28.4 ± 1.41), and significantly higher in lactose (53.6 ± 1.98) than in glucose, maltose, and sucrose solutions (34.3 ± 1.50, 31.8 ± 1.42, 34.5 ± 1.65, respectively). Although the concentrations of sweeteners we used were selected to be at threshold concentrations about equivalent to 0.5% sucrose solutions, different research methods lead to different sweetness equivalencies (Shallenberger, 1982). Lactose was reported as 16% as sweet as sucrose by Ockerman (1975), 16–48% as sweet by Shallenberger and Acree (1971), and 16–30% as sweet by Tver and Russell (1989). We found that sweetness of lactose was at the higher end of the sucrose equivalency range. The initial lower sweetness of aspartame samples might also be due to some degradation during heating, although conditions were not severe. According to the manufacturer, aspartame should remain stable during the heating conditions used.

Overall differences in sweetness partitioned by gum type

The mean sweetness intensity for all sweeteners in aqueous solutions increased with added NaCl, with exception of maltose

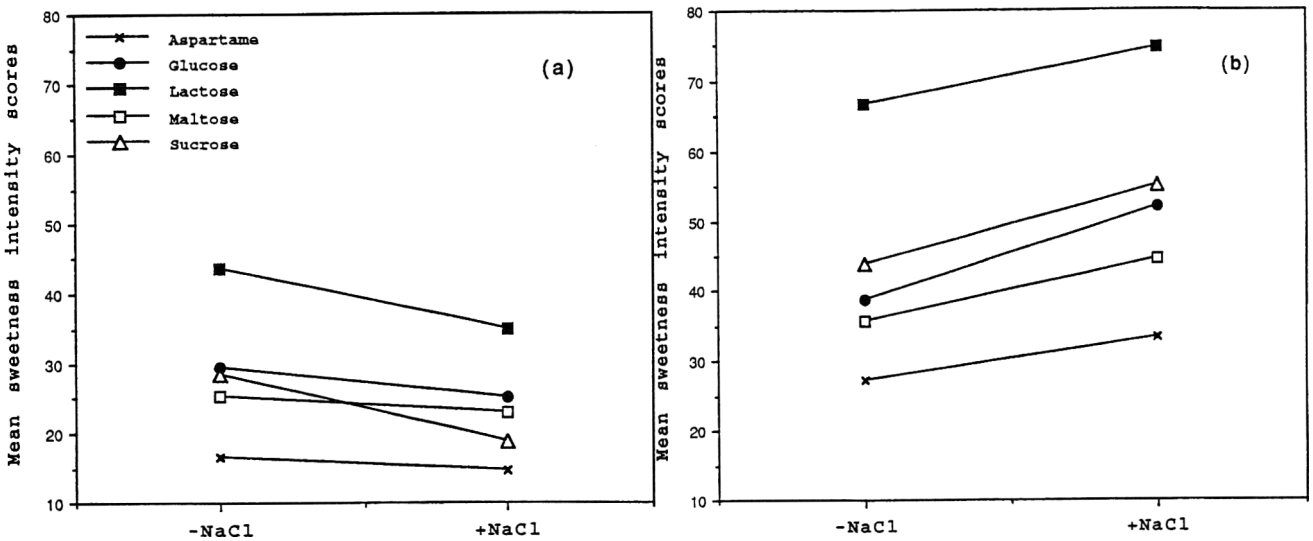

Fig. 3—Mean sweetness intensity scores for sweeteners in (a) κ-carrageenan and (b) xanthan solutions with (+NaCl) and without (−NaCl) NaCl. (n = 39: 13 subjects × 3 reps).

(Fig. 1). The added NaCl, together with the inherent Na^+ in the sweeteners may have slightly enhanced sweet taste, although the effect was only significant at p = 0.0780. Previous studies of enhancement of sweetness by dilute NaCl used higher concentrations of both NaCl and sugars (Fabian and Blum, 1942; Bartoshuk, 1975) and showed slight effects. Differences between studies could be attributed to the degree of the effect, concentrations of solutes, and/or sensory method differences.

Because there were significant differences in sweetness between solutions made with the different gums, the data were partitioned by gum type. The overall effect of sweetener type on sweetness was also observed when partitioned by gum type. The most obvious increase in sweet taste occurred in lactose solutions, all of which were judged initially sweeter than other sweetener solutions. Thus, a slightly higher level of the other sweeteners may possibly cause similar increases in sweetness as those in lactose solutions.

For all sweeteners in guar solutions, the most obvious increase in sweetness was in the lactose/guar solutions (Fig. 2a). The sweetness of aspartame, glucose, lactose, and maltose increased with addition of NaCl in locust bean solutions (Fig. 2b) while sweetness of sucrose decreased slightly. Overall, the addition of NaCl did not affect sweetness in guar solutions (p = 0.1474) nor locust bean solutions (p = 0.0907).

Sweetness of all solutions containing κ-carrageenan decreased (p = 0.0253) with added NaCl (Fig. 3a). We did not expect these results, which might be attributed to masking of sweet taste by saltiness. However, locust bean solutions were as salty as κ-carrageenan, and no masking of sweetness was seen. Mean sweetness intensity of xanthan solutions increased (p = 0.0082) with addition of NaCl (Fig. 3b). This may be explained by the inherently sweeter taste of xanthan. Perhaps a minimum sweetness, equivalent to that of xanthan or lactose solutions was necessary for sweet taste to be enhanced by NaCl.

Correlations of sweetness with other attributes

Correlations showed that sourness was highly associated with sweetness for all sweeteners tested, with exception of lactose. Since lactose was sweeter than the other solutions, it may have masked the sour taste. High associations may be due to the acidic pH of many of the solutions (pH range 3.75–7.05). It may also be due to the close range of apparent specific volumes necessary to elicit sour and sweet tastes (≈0.33–0.52, 0.52–0.71, respectively) (Shamil et al., 1987). Aspartame solutions had strong correlations of metallic (p = 0.0211) and sour (p = 0.0003) with sweetness, but bitter taste was not significantly correlated with sweet. Both maltose (p = 0.0027) and sucrose (p = 0.0048) showed correlations between sweet and metallic taste. Thickness correlated with sweetness for all sweeteners. Xanthan solutions were thickest and sweetest and κ-carrageenan solutions were thinnest and least sweet. Saltiness correlated negatively with sweetness (p = 0.0063) for lactose, but no other correlations with saltiness were noted.

Factors affecting saltiness

Saltiness intensity values showed effects for gum type (p = 0.0001), added NaCl (p = 0.0001), and gum-NaCl interaction (p = 0.0001), as well as sweetener type (p = 0.0420), and sweetener-NaCl interaction (p = 0.0004). Mean saltiness intensities ± standard error of the mean (n = 390) were lower in xanthan solutions (23.4 ± 1.38) than in aqueous (29.7 ± 1.67), guar (28.4 ± 1.31), locust bean (30.9 ± 1.64), and κ-carrageenan (29.7 ± 1.58), which did not differ. The lower saltiness intensities of xanthan solutions confirmed our earlier results (Rosett et al., 1994, 1995), indicating that Na^+ was more "bound" and unavailable for salt taste. Additionally, this may be due to the initially sweeter taste of xanthan solutions compared to other gum solutions, since the general effect in taste mixtures is masking (Pangborn, 1974; Bartoshuk, 1975). In the pilot study, we observed that sweetness thresholds were lower in sucrose/xanthan solutions than in other gum solutions, indicating that xanthan was inherently sweeter than the other gums.

Overall mean saltiness (n = 390) was higher in sucrose solutions (32.2 ± 1.64) than in aspartame (27.2 ± 1.49), maltose (26.6 ± 1.48), and lactose (26.5 ± 1.65), with glucose intermediate (29.6 ± 1.58). Sucrose solutions were also perceived as less sweet compared to other sweetener solutions, which could be due to masking by the salty taste. Overall mean saltiness intensities were higher (p = 0.0001) in solutions with added NaCl than without added NaCl, as expected. Gum-NaCl and sweetener-NaCl interactions were significant, indicating that although solutions increased overall in sweetness with added NaCl, the effect was not evident in all systems.

Sweetener type affected saltiness in aqueous (p = 0.0359) and locust bean (p = 0.0483) solutions, but did not affect κ-carrageenan (p = 0.0624), xanthan (p = 0.2383), and guar (p = 0.3736) solutions. Sweetener-NaCl interaction was significant in aqueous (p = 0.0012), guar (p = 0.0080), and locust bean (p = 0.0128). Although mean saltiness increased with added NaCl in all solutions tested, the extent and direction of the effect varied.

Na^+ binding and sweet taste

Since R_2 values for guar and locust bean solutions were similar to κ-carrageenan, Na^+ "binding" or mobility was similar. This indicates that κ-carrageenan behaved as a non-ionic gum, possibly due to the presence of high levels of Ca^{2+} and K^+. If the mobility of Na^+ affects sweetness, as well as saltiness, comparable effects might be expected with these gums. However, κ-carrageenan solutions with 0.05% added NaCl were perceived as less sweet than those without added NaCl for all sweeteners tested, possibly due to unique taste qualities exhibited by κ-carrageenan, as well as the presence of other cations (Ca^{2+} and K^+). Mean sweetness values with and without added NaCl for κ-carrageenan (Fig. 3a) were much lower than the values for aqueous (Fig. 1), guar (Fig. 2a), locust bean (Fig. 2b), and xanthan (Fig. 3b) solutions. Thus, other taste attributes may have masked sweetness in κ-carrageenan solutions without added NaCl, so that the level of sweetness needed for sweet taste enhancement with added NaCl was not reached.

R_2 values for xanthan indicated that much of the Na^+ was "bound" in solutions containing 0.05% added NaCl. Since xanthan contains less Ca^{2+} (4.332 ppm) and K^+ (115.026 ppm) than κ-carrageenan, there would be available sites for Na^+ to bind. Thus, the total Na^+ (added + endogenous) left mobile may have been low enough to enhance sweet taste without contributing saltiness. This would explain the increased sweet taste with added NaCl in xanthan solutions.

Added NaCl and gum type appeared to affect sweetness of some systems. Enhancement of sweeteners by NaCl was more apparent with higher initial sweetness. Thus, a minimum amount of sweetening agent must be present before addition of NaCl could have an effect. Other cations (i.e., K^+ and Ca^{2+}) displace Na^+ and allow it to remain free to start the sweet taste transduction cascade, thus enhancing sweetness. Studies in mice by Tonosaki (1992) suggested that the binding of each taste stimulus (salt or sweetener) to the taste cell receptor activated internal second messengers. Enhancement or masking could then occur, activated by the different stimuli and their interactions. Thus, changes in binding of Na^+ by gums may either enhance or mask saltiness or sweetness. Our results provide indirect evidence to support the hypothesis that Na^+ is involved in a secondary pathway enhancing sweetness.

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Rheological Properties of Solutions and Emulsions Stabilized with Xanthan Gum and Propylene Glycol Alginate

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ABSTRACT

The rheological effects of propylene glycol alginate (PGA) added to solutions and model emulsions containing xanthan gum (XG) were studied using controlled stress rheometry with concentrations appropriate for salad dressings. For samples with XG and XG + PGA blends, solutions and emulsions showed a Newtonian plateau at low shear stresses. The Newtonian plateau of a solution accurately predicted (r^2 = 1.00) the Newtonian plateau for an emulsion of equivalent gum concentration. Addition of PGA to constant levels of XG showed a more than additive increase in the Newtonian plateau viscosity for solutions and emulsions. For XG aqueous solutions, pseudoplasticity decreased upon addition of PGA. Storage and loss moduli increased with addition of PGA to XG for solutions and emulsions, although G' for solutions of PGA alone were negligible.

Key Words: propylene glycol, rheology, viscosity, alginate, xanthan gum

INTRODUCTION

XANTHAN GUM and propylene glycol alginate are commonly used as blends in salad dressings. Most full and low fat retail dressings (other than thousand island) are stabilized with XG. Many are also stabilized with PGA. The rheological contributions of PGA when blended with XG have not been clearly documented. King (1982) suggested that dressings stabilized with PGA in conjunction with XG produced a "longer" flow than those produced with XG alone. However, rheological and sensory data were not published. Studies of solutions and emulsions including XG and PGA in combination with other ingredients have been reported (Fishbach and Kokini, 1987; Coia and Stauffer, 1987, Carrillo and Kokini, 1988; Yilmazer and Kokini, 1991; Yilmazer, et al., 1991). However, few have focused specifically on the rheological effects of adding PGA to XG solutions and emulsions.

Xanthan gum was approved for general use as a food additive in the US in 1969 and then approved by other international regulatory agencies during following years. Xanthan gum, produced by fermentation of *Xanthamonas campestris*, is a high molecular weight, water soluble heteropolysaccharide. Milas et al. (1990) reported the average molecular weight of xanthan gum was 5.25×10^6 Daltons while others (Bezemer et al., 1993) have reported the molecular weight to be $\sim 10^7$ Daltons.

Xanthan gum imparts unique rheological properties to solutions. Under low shear conditions XG displays a high solution viscosity; however sharp reductions in viscosity occur under high shear (Sanderson, 1981; Milas et al., 1990). The pseudoplastic behavior of XG, along with its pH and temperature stability (Kang and Pettitt, 1993), make it an ideal thickener for salad dressings. Those dressings containing XG pour readily from a bottle and then recover their initial high viscosity at rest. As a result, such dressings cling or adhere to ingredients (Sanderson, 1981). Xanthan gum imparts body to oil in water (O/W) emulsions (Coia and Stauffer, 1987).

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Propylene glycol alginate is a derivative of alginic acid with an average molecular weight ranging from 30,000 to 200,000 Daltons (McNeely and Pettitt, 1973). In contrast to XG, PGA displays a low degree of pseudoplasticity in solution (Coia and Stauffer, 1987) and promoted creaminess without significant rheological changes. Other researchers reported that when XG was partially replaced by PGA, emulsions were more fluid-like and exhibited reduced viscosity (Yilmazer et al., 1991; Yilmazer and Kokini, 1991).

Our objectives were to further characterize the rheological contributions of PGA to solutions and O/W emulsions containing XG, to study the very low shear stress range for a yield point, to evaluate effects of salt and pH and to determine if aqueous solution rheology would predict emulsion rheology.

MATERIALS & METHODS

XG, PGA AND BLENDS were evaluated in three media using concentrations relevant to full calorie salad dressings. First evaluations were made in simple aqueous media. Secondly, sodium chloride and acetic acid were added to aqueous media at levels appropriate to salad dressings. Finally, the gums were evaluated in emulsions that modeled full calorie (40% oil), pourable, ranch dressing.

Aqueous samples

Samples of xanthan gum (food grade, Archer Daniels Midland, Decatur, IL) and propylene glycol alginate (Kimiloid LVC, Kimitsu Chemical Industries, Japan) were used without further purification or modification. Total solids were determined by drying for 2.5 hr at 105°C in a vacuum oven, and samples were prepared at concentrations corrected to 100% total solids. Gums were initially hydrated in "standardized tap water" (STW). STW was prepared as 1.0g/L NaCl and 0.15g/L $CaCl_2 2H_2O$ (Clark, 1987). Solutions of each gum at 1.0% were prepared by sifting gum directly onto the water vortex in a three-bladed propeller type mixer, and all solutions were allowed to mix for 60 min. Solutions were further diluted and blended as required from the 1.0% solutions and continued mixing an additional 10 min. Solutions were then degassed and tested the following day. Two replicates were prepared at each concentration and each replicate was tested twice (n = 4). For aqueous solutions designed to model the salt and acid conditions of salad dressings (pH = 3.8 and NaCl = 1.5%), gums were first hydrated in STW and then subsequently adjusted to final specification.

Model emulsions

The emulsions were designed to model full calorie, pourable, ranch dressings. Model emulsions were prepared from (% w/w) STW (44.8) vegetable oil (Wesson™ soybean oil), (40.0) cultured buttermilk solids (4.5) (Land O'Lakes™), acetic acid (3,3) sugar (3.0) egg yolk (2.5), and salt 1.5%. Variations in gum weights were accounted for by corresponding adjustments in water. Pre-emulsification was done with a three-bladed propeller type mixer. Final emulsification was done using a Rannie high pressure lab homogenizer (Mini-Lab, Type 8.30-H). Samples were passed through the homogenizer one time using a pressure of 200 bar. Two replicates were prepared at each concentration and each replicate was tested once (n = 2). Samples were stored at room temperature for 24 hr before rheological evaluation. Oil droplet size (mean volume diameter) was determined by light scattering using a Malvern Mastersizer MS20 from Malvern, England (focal length 45mm; range: 0.1–80 μm). Samples were prepared for analysis by making a dilute suspension of emulsified particles in 0.9% (w/v) NaCl solution.

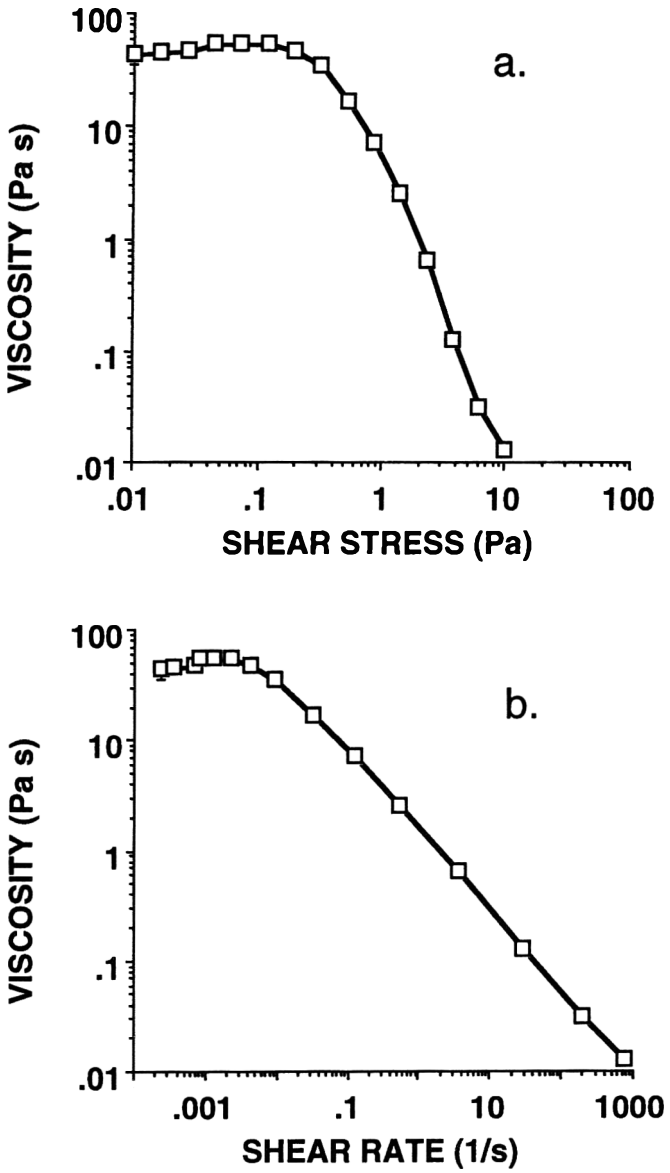

Fig. 1—Viscosity relationship to a) shear stress and b) shear rate for a 0.33% xanthan gum (XG) solution in "Standardized Tap Water" (STW).

Table 1—Gum concentrations (w/w) used in aqueous solutions and emulsions (40% oil) of equivalent aqueous phase gum concentrations

Solutions (XG% + PGA%)	Model emulsions (XG% + PGA%)
—	0.00 + 0.00
0.00 + 0.33	—
0.00 + 0.55	0.00 + 0.25
0.33 + 0.00	0.15 + 0.00
0.33 + 0.33	0.15 + 0.15
0.33 + 0.55	0.15 + 0.25

All gum concentrations were reported as concentrations in the aqueous phase, rather than total weight percent. Due to the high oil content of full calorie salad dressings and the fact that hydrocolloids are present only in the aqueous phase of an emulsion, gum concentration in the aqueous phase of each emulsion was actually much higher than the total weight percent indicated. For example, in this study an emulsion at 0.15% XG (total weight percent) is actually 0.33% in the aqueous phase. Consequently, gum concentrations in all aqueous samples were increased to the target aqueous concentrations in model emulsions. For simplicity, gum concentrations in model emulsions were reported in terms of aqueous concentrations (Table 1).

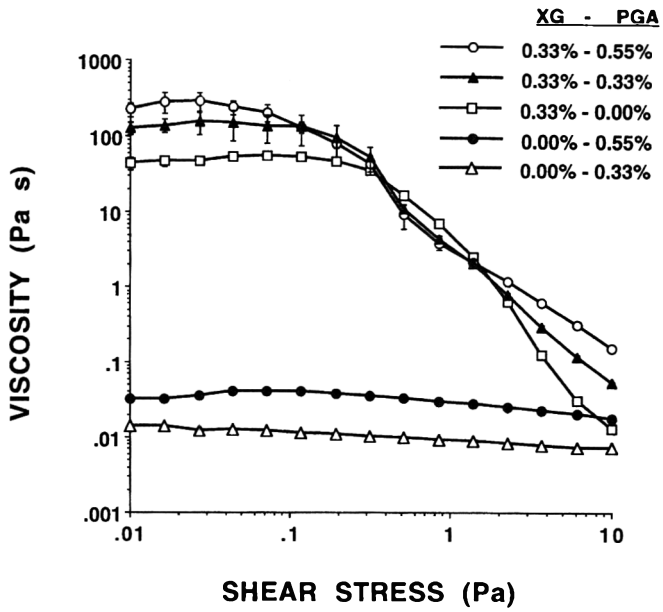

Fig. 2—Effect of added propylene glycol alginate (PGA) on viscosity of aqueous solutions of XG in STW.

Rheological measurements

A controlled stress rheometer (Carri-Med CSR Model CSL 100, T.A. Instruments, New Castle, DE), using Carri-Med software (version 5.3), was used for dynamic oscillatory measurements and flow curves. The measurement geometry was a 6 cm cone and plate with a cone angle of 0.0346 radians. Frequency sweeps from 0.063 to 62.83 radians sec^{-1} were performed on aqueous samples and emulsions after establishing the linear viscoelastic range. For aqueous samples this was between 0.0177 and 0.177 Pa and for emulsions it was between 0.177 and 1.77 Pa. The shear stresses used for frequency sweeps of aqueous samples and emulsions were 0.133 and 1.24 Pa, respectively. To prevent dehydration a thin layer of a low viscosity silicon oil (Fisher Scientific) was applied to the exposed perimeter of all samples prior to rheological evaluation. In order to avoid time dependent rheological effects, each data point collected for development of flow curves was generated by applying a fixed stress to the sample and monitoring the shear rate until equilibrium was reached. Shear rate and viscosity were then recorded. Flow properties for the emulsions were evaluated as described except the rheometer was programmed to pause 30 min between sample loading and initiation of rheological measurements in order to allow samples to recover.

RESULTS & DISCUSSION

Steady shear viscosity results

Aqueous solutions. For all aqueous solutions, 15 shear stresses were applied over three decades, from 0.01 to 10 Pa. Due to the pseudoplasticity and high viscosity at low shear rates of the XG solutions, three decades of shear stress (Fig. 1a) corresponded to six to seven decades of shear rate (Fig. 1b). As a result, controlled stress rheometry provided a method for obtaining viscosity data at shear rates lower than those obtainable from controlled shear rate instruments and therefore provided a better test for yield stress (Barnes and Walter, 1985).

For aqueous solutions containing XG, experimental measurements suggest the presence of a low shear stress Newtonian plateau, or limiting viscosity, rather than a yield stress (Fig. 1a and 1b). The viscosity curves partially fit a generalized model (Barnes et al., 1989) composed of several regions. At low shear rates or stresses a suspension behaves as a Newtonian fluid (η_o). At intermediate shear rates or stresses a suspension behaves as a power law fluid. At very high shear rates or stresses, a suspension behaves as a Newtonian fluid (η). Measurements of the XG and XG + PGA solutions showed Newtonian plateaus at low shear stresses and power law regions at higher shear

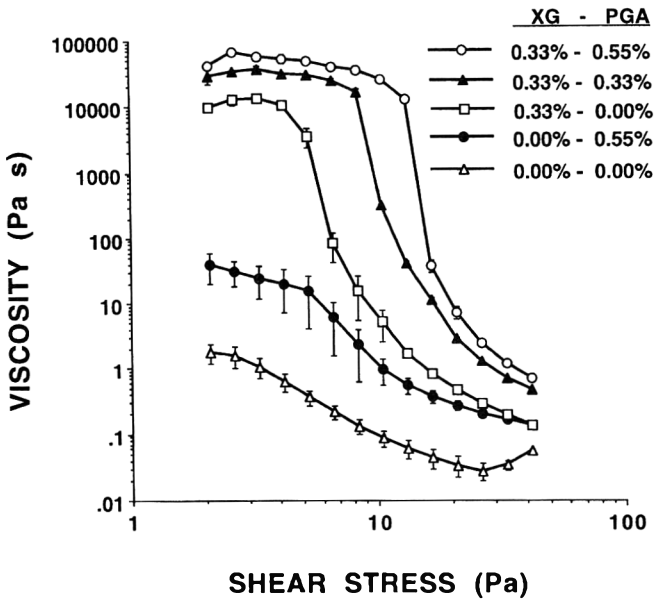

Fig. 3—Effect of added PGA on viscosities of O/W emulsions (40% oil).

Table 2—Low shear viscosity of solutions and emulsions. Particle size was calculated as mean volume diameter

Gum concentrations[a] (XG% + PGA%)	Aqueous viscosity (Pa·s) (n=4)	Emulsion viscosity (Pa·s) (n=2)	Emulsion particle size (μm)
0.00 + 0.00	—	1.4 ± 0.12	10.17 ± 1.8
0.00 + 0.55	0.04 ± 0.002	25 ± 7	10.61 ± 0.01
0.33 + 0.00	50 ± 5	10,000 ± 900	3.00 ± 0.66
0.33 + 0.33	145 ± 12	28,000 ± 2800	2.93 ± 0.12
0.33 + 0.55	250 ± 36	48,100 ± 7200	2.43 ± 0.61

[a] In the aqueous phase.

Table 3—Power law parameters K and n (consistency index and flow behavior index) for shear thinning region of aqueous solutions (Fig. 2) and emulsions (Fig. 4) with xanthan gum and propylene glycol alginate[a]

Gum concentration[a] (XG + PGA) %	Aqueous solutions (STW) K	n	Emulsions (40% oil) K	n
0.00 + 0.00	—	—	1.738	0.3657
0.00 + 0.33	0.0144	0.9040	—	—
0.00 + 0.55	0.0474	0.8554	3.541	0.2707
0.33 + 0.00	1.6340	0.2485	5.476	0.3359
0.33 + 0.33	1.5530	0.3523	11.135	0.2707
0.33 + 0.55	1.6800	0.4353	14.957	0.2255

[a] (Shear Stress) = K (Shear Rate)^n
[b] In the aqueous phase.

stresses. A high shear Newtonian region was not found for any of our solutions.

Researchers have suggested that XG stabilizes O/W emulsions by imparting a yield stress to the aqueous phase (Hennock et al., 1984). However, our results confirmed Milas et al. (1990) who showed a Newtonian plateau at low shear rates or stresses for XG solutions. Identification and quantification of the low shear viscosity plateau of the aqueous phase should be useful for prediction of emulsion stability. Shear rates and stresses experienced by oil droplets undergoing gravitational separation or flocculation would be in this low shear rate range.

Addition of PGA to a 0.33% XG solution had several effects. The first was considerable increase in the low shear Newtonian plateau relative to XG alone (Fig. 2). There were around 3- and 5-fold increases in limiting viscosities with addition of 0.33 and 0.55% PGA to XG, respectively (Table 2). Since the solution of 0.33% XG had a viscosity about 1000 times greater than a

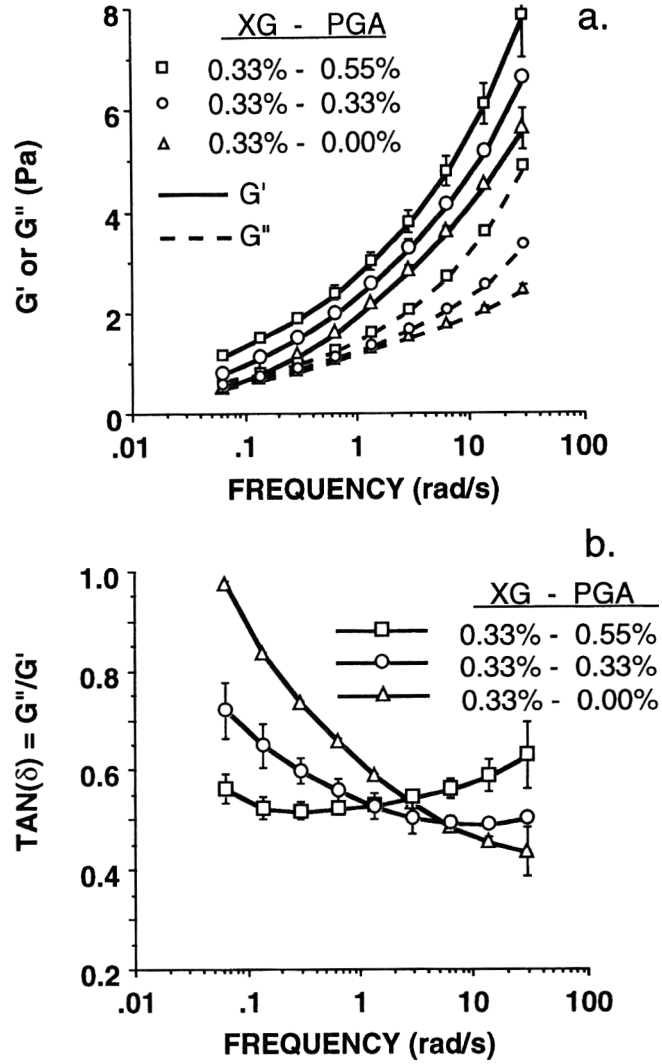

Fig. 4—Effect of added PGA on viscoelasticity of aqueous solutions of XG in STW, (a) the storage modulus G' and loss modulus G" and (b) tan δ (G"/G').

solution of 0.55% PGA alone, the fivefold increase in viscosity of the XG + PGA solution over the XG solution alone was not expected. At an intermediate stress range around 1 Pa, PGA had no additive effect on viscosity of XG alone. The XG + PGA solutions had slightly lower viscosities than the XG solution. At higher shear stresses the solutions had the same ordering of viscosities as in the Newtonian plateau region. The second observed effect of adding PGA to XG was to lessen the pseudoplasticity of the XG solution (Table 3). Without sensory data the effect of such rheological profiles on perception of texture is not known.

Aqueous solutions at 0.33% XG and 0.33 XG + 0.55% PGA were also prepared at salt and acid levels appropriate for salad dressings (NaCl = 1.5%, pH = 3.8). This change in ionic strength and pH did not produce changes in viscosity profiles at constant gum levels. This suggested that the effects of PGA addition to XG were not moderated under the aqueous conditions of a salad dressing as was the case with guar gum and XG (Clark, 1987).

Emulsions

Like the aqueous gum solutions, flow curves for emulsions stabilized with XG showed the presence of a limiting viscosity or Newtonian plateau at low shear stresses (Fig. 3). A sharp increase in limiting viscosity was observed upon addition of XG

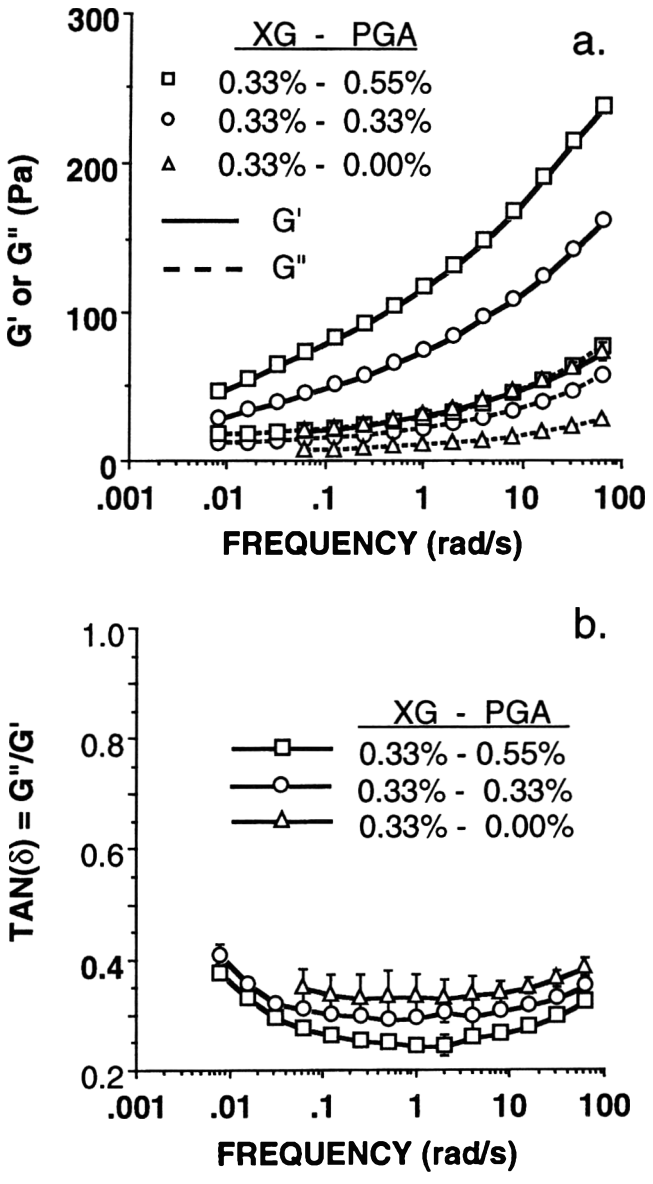

Fig. 5—Effect of added PGA on viscoelasticity of O/W emulsions made with 40% oil, (a) the storage modulus G' and loss modulus G" and (b) tan δ(G"/G').

alone as compared to the control emulsion without gum. This viscosity building effect of XG has been well documented (Anonymous, 1988). Emulsions made with PGA alone compared to the control showed some degree of enhanced viscosity. However, the effect was small compared to emulsions made with XG alone, as expected. Emulsions with 0.33% XG had a viscosity about 2500 times greater than an emulsion containing only 0.55% PGA.

Note that oil droplet sizes were not constant for these emulsions. Samples made without XG had particle sizes larger than those made with XG (Table 2). These results confirmed previous work by Hennock et al., 1984. This was a contributing factor for the lower viscosities of the samples made with PGA alone, but was not a factor when comparing XG alone to XG + PGA emulsions.

Emulsions formulated with blends of XG + PGA showed considerable increase in Newtonian plateau viscosity compared to the emulsion made with XG alone. About three and fivefold increases occurred in viscosity with the addition of 0.33 and 0.55% PGA to the 0.33% XG, respectively (Table 2). These results were predicted by plateau viscosities of the aqueous solutions (r^2 = 1.00).

Unlike the aqueous solutions, viscosities of emulsions with XG and XG + PGA did not converge at shear stresses greater than the Newtonian plateau (Fig. 3). In addition, the viscosities of emulsions containing XG broke down more rapidly with increasing shear stress beyond the Newtonian plateau. This rapid breakdown may be viewed as similar to a yield stress, except for the existence of a limiting viscosity rather than an infinite viscosity at zero or low shear rates. In this case the application of shear stresses which were within the Newtonian plateau produced small deformations which were within the linear viscoelastic range. Consequently, the viscosity measured at equilibrium flow corresponded to the Newtonian compliance in a model for food emulsions proposed by Sherman (1967). As long as the applied shear stress was within the linear viscoelastic range of the emulsion, the measured equilibrium viscosity was constant (Newtonian plateau). When values of stress exceeded the linear range, a rapid decline in equilibrium viscosity was observed (Fig. 3). This behavior also corresponded to flow curves for the aqueous gum solutions except the decline of viscosity at stresses exceeding the linear range was not as rapid as for emulsions. In either case our observed "yield stress" corresponded to the upper limit of the linear viscoelastic range of the solution or emulsion.

Viscoelasticity

Aqueous solutions. The effect of added PGA on the viscoelasticity of aqueous XG and XG + PGA solutions was measured with small shear stress oscillatory experiments. The storage modulus, G', increased with added PGA (Fig. 4a) though the G' for PGA alone was so small that it was not measurable. Storage moduli for the three solutions increased at the same apparent rate, indicating that relative differences in elasticity among solutions remained constant. There appeared to be a more divergent behavior among the loss moduli with increasing frequency. This is better illustrated in a plot of tan δ(G"/G') vs frequency (Fig. 4b). Tan δ is a measure of the ratio of viscous to elastic (G"/G') properties of a material. At low frequencies, the addition of PGA to XG solutions caused a decrease in tan δ. However, at the high end of the frequency range, this relationship was reversed. This indicated an increase in the viscous over the elastic nature with increasing frequency for solutions of added PGA.

Emulsions. As with aqueous samples, addition of PGA to XG emulsions caused G' to increase (Fig. 5a). However, addition of PGA to the emulsion did not have the same effect on tan δ, the relative degree of viscoelasticity (Fig. 5b) as was observed in aqueous solutions In this case, variations in tan δ with frequency were similar with and without PGA. Possibly, the elasticity of the oil droplets suspended in the aqueous phase dominated the measured viscoelasticity and therefore masked the viscoelastic effects of added PGA.

CONCLUSIONS

USE OF A CONTROLLED STRESS RHEOMETER demonstrated the occurrence of a low shear stress Newtonian plateau, or limiting viscosity, rather than a yield stress for both solutions and emulsions stabilized with XG and XG + PGA. Solution rheology predicted emulsion rheology well at very low shear rates (r^2 = 1.00), but not beyond the limiting viscosity range, and not in viscoelastic data. Adjusting pH and salt concentrations as tested did not significantly change solution rheology. A modified definition of yield stress is proposed for such samples based on stress values that are outside the linear viscoelastic range. This leads to a rapid "yielding" or precipitous decrease in apparent viscosity, especially for emulsions.

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—Continued on page 550

Polymerization and Mechanical Degradation Kinetics of Gluten and Glutenin at Extruder Melt-Section Temperatures and Shear Rates

T.D. STRECKER, R.P. CAVALIERI, R.L. ZOLLARS, and Y. POMERANZ

ABSTRACT

Thermal polymerization and mechanical degradation rates were measured for wheat gluten and glutenin at 25–30% moisture. Changes in soluble protein and disulfide bonds were measured at 85°C to 180°C, residence times of 2–60 sec and shear rates of 10–275 sec^{-1}. Polymerization rates without shear were evaluated using isothermal analysis. An extrusion rheometer was used to simulate extruder conditions to determine the combined polymerization-degradation rates. Molecular weight distributions of soluble reaction products were determined using liquid size exclusion chromatography. Activation energies were 0.60–2.1 kcal/mol, 2.0–8.2 kcal/mol, and 0.50–0.90 kcal/mol and reaction orders were 0.5–2.0, 0.8–1.5, and 0.6–0.7, respectively for polymerization without shear, polymerization with shear, and degradation.

Key Words: wheat, gluten, glutenin, polymerization, extrusion

INTRODUCTION

EXTRUSION-COOKED PRODUCTS such as modified starches, cereals, snack foods, textured plant protein, etc. are common in the food industry (Harper, 1981a) and wheat flour and gluten are important feedstocks (Barres et al., 1990; Lawton et al., 1985; Boyal and Phoolka, 1989; Andersson and Hedlund, 1990). Studies have been reported on extrusion of cornmeal (Bhattacharya and Hanna, 1986), soy flour (Baird and Reed, 1989; Wagner, 1987; Morgan, 1979; Remsen and Clark, 1978) and wheat starch (Davidson et al., 1984; Diosady et al., 1985). They have included transport processes (Baird and Reed, 1989; Wagner, 1987), rheology (Morgan, 1979; Remsen and Clark, 1978), reaction kinetics (Davidson et al., 1984; Wang et al., 1992; Bhattacharya and Hanna, 1987), physical (Wagner, 1987; Morgan, 1979), and functional properties (Stanley, 1989). The importance of chemical and physical reactions on mass transport and heat transfer during extrusion has been recognized, but relatively few kinetic studies have been reported. Bhattacharya and Hanna (1987) determined zero-order kinetics for chemical reactions during cornmeal extrusion. Wang et al. (1992) also observed zero-order kinetics for changes in waxy corn starch at extruder temperatures and moistures. Davidson et al. (1984) reported first-order kinetics for wheat starch degradation in a single-screw extruder. Others (Suzuki et al., 1976; Kubota et al., 1979) determined gelatinization kinetics of rice and potato starch. The importance of chemical reactions in both starch and protein fractions of commonly extruded food materials has been recognized (Remsen and Clark, 1978; Morgan, 1979; Stanley, 1989), but most studies have focused on the starch fraction. Little work has been reported elucidating the chemical reactions occurring in proteins during extrusion.

Wheat flour has a unique composition compared to other feedstocks. Though protein comprises 10–14% (wt) of the total flour, it is responsible for the viscosity and elasticity of dough to a great extent (Greenwood and Ewart, 1975; Funt Bar-David and Lerchenthal, 1975). Gluten is comprised of glutenin and gliadin. Glutenin, a linear, asymmetric protein with molecular weights ranging > a million Daltons, is responsible for the viscoelastic properties of gluten (Greenwood and Ewart, 1975). Pence et al. (1953) reported the denaturation rate of gluten and the gliadin fraction at 80–90°C and 5–60 min reaction times.

Ewart (1968, 1972, 1979, 1988) determined the influence of disulfide bonds on gluten viscoelastic properties and structure. Glutenin was hypothesized as a trifunctional monomer capable of polymerizing via disulfide bond formation. Schofield et al. (1983) analyzed the effects of heat on sulfhydryl-disulfide interchange reactions and concluded they were the primary bonds responsible for network formation upon thermosetting. Other researchers (Kaczkowski and Mieleszko, 1980; Beckwith and Wall, 1966; Graveland et al., 1978) have supported the disulfide bond hypothesis, but extensive research will be required to fully understand the complex mechanisms involved.

Our objective was to determine polymerization and mechanical degradation rates of gluten and glutenin based on the following step-growth polymerization mechanisms:

$$M_1 + M_1 \xrightarrow{k_p} P_2 \quad (1)$$

$$M_1 + P_2 \xrightarrow{k_p} P_3 \quad (2)$$

$$\vdots$$

$$M_1 + P_{n-1} \xrightarrow{k_p} P_n \quad (3)$$

$$P_n + P_m \xrightarrow{k_p} P_{n+m} \quad (4)$$

$$P_{n+m} \xrightarrow{k_d} P_r + P_s \quad (5)$$

where M_1 is monomer; P_i is polymer length 2, 3, n − 1, n, m, n + m, r, or s; k_p is the polymerization rate constant; and k_d is the degradation rate constant.

Assumptions of the model were: no diffusional effects as molecular weight increased, equal reactivity between thiol groups, and the reaction between thiol groups was a reduction/oxidation mechanism.

MATERIALS & METHODS

Fractionation

Commercial gluten, purchased from Midwest Grain Company, Atchison, Kansas, was fractionated into ethanol soluble gliadin and ethanol insoluble glutenin (Osborne, 1907). The water soluble proteins, albumin and globulin, were solubilized by mixing gluten and 0.5M NaCl, agitating 2 hr at 4°C, followed by centrifugation for 2 hr at 2000 × *g*. The supernatant was discarded and the pellet rinsed 30 min with 40 mL distilled water to remove residual salt. The washed pellet was then mixed with 70% ethanol for 2 hr at 25°C followed by centrifugation for 1 hr at 2000 × *g*. The supernatant (gliadin) and the pellet (glutenin) were freeze-dried and stored at 4°C. Each powdered sample was hydrated to

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25% or 30% moisture (w.b.) and stored overnight at 4°C to reach equilibrium.

Experimental procedures

Gluten and glutenin samples were equilibrated to room temperature (≈23°C) prior to polymerization and degradation rate studies. All studies were conducted in triplicate. Polymerization rate studies without shear were conducted by positioning samples (1 g) between two 8 mm thick stainless steel plates heated to 190 ± 0.5°C. Quick-release clamps pressurized samples to prevent steam from flashing. Thermal reactions were quenched using liquid nitrogen and temperature of proteins between the plates was measured (Fig. 1). A thermocouple (T-type, 40 ga.) was embedded in the center of a sample to monitor temperature rise. Data are averages of six experimental values.

To reach 170–175°C required a heating time of 4–10 sec independent of moisture in the 25–30% range. Heating times of 10–60 sec produced extensive browning and burning near 60 sec. Residence times of 4–10 sec at 170–175°C are typical of the melt section of a single-screw extruder (Harper, 1981b). The <1 mm thick sample rapidly reached reaction temperatures ensuring accuracy of kinetic data. Polymerization was evaluated through analysis of solubility and disulfide bonds.

An extrusion rheometer was used to apply combined heat and shear to samples. A typical extrusion rheometer, or capillary rheometer, consists of a short die (1.27 cm) attached to a heated sample reservoir. In this type rheometer the sample is allowed to equilibrate to temperature and subsequently extruded by a plunger forced downward by an Instron Universal Testing apparatus. Materials are allowed to complete reactions before extruding. This procedure measures the post-process rather than in-process viscosity. However, to simulate a reacting flow such as occurs in an extruder melt section, an extrusion rheometer must allow reactants to react and flow simultaneously. The apparatus to do this (Fig. 2) consisted of a 10.2 cm long and 1.59 mm diameter capillary. Temperatures of 26, 110, 167, and 209°C were used and residence times were 2–60 sec. These were comparable to residence times in an extruder melt section (Harper, 1981b). The <40°C sample reservoir ensured that no reactions occurred before extrudate entered the heated die region. Test samples were 5g and extrusion force at specific extrudate flow was measured. Gluten and glutenin extrudates were analyzed for disulfide bond and soluble protein concentration. Extrudate temperatures were measured via a thermocouple probe inserted into the die. A 1 mm diam × 30 cm long probe was inserted into the pressure vessel attached to the bottom of the rheometer through a probe seal (Omega) and threaded up the die to contact the sample in the cool sample reservoir. The probe was subsequently allowed to move down the die with the extrudate to measure the temperature along the die. Nonisothermal data were analyzed using nonisothermal techniques. Shear stress and shear rates were determined from pressure and velocity. Shear stresses ranged from 1.0–9.5 M Pa and shear rates ranged from 10–275 sec^{-1}. Initial studies to determine experimental variability showed coefficients of variation (CV) ≈ 3%.

Extrudate samples were immediately frozen, freeze dried, and milled using a Udy mill equipped with a 0.5 mm screen. Powdered samples were stored at 4°C in individual plastic ziplock bags until analyzed.

Assays

Three assays were performed on each sample. Soluble protein was determined by a modified Lowry protein assay (BioRad) on an 8M urea, 0.2M Tris and 0.5% SDS extract (soluble protein was related to extent of polymerization). A free thiol and total sulfhydryl assay was employed to determine disulfide concentration (Chan and Wasserman, 1993). It has been hypothesized that gluten and glutenin crosslink via disulfide bonds (Ewart, 1988; Graveland et al., 1978) Therefore, disulfide bond concentration was used as an index of extent of reaction.

Size exclusion chromatography

Size exclusion chromatography (SEC) was used to determine molecular weight distribution and soluble product concentrations using protein standards with molecular weights ranging from 29,000–2,000,000. The mobile phase was 0.1M sodium phosphate buffer, pH 6.9, with 0.1% sodium dodecyl sulfate (SDS). The column (30 cm × 7.5 mm) used was a Varian TSK 4000SW silica gel (13 μm diameter and 450 Å pore size) preceded by a guard column. The eluent was monitored by a 210 nm UV absorbance detector.

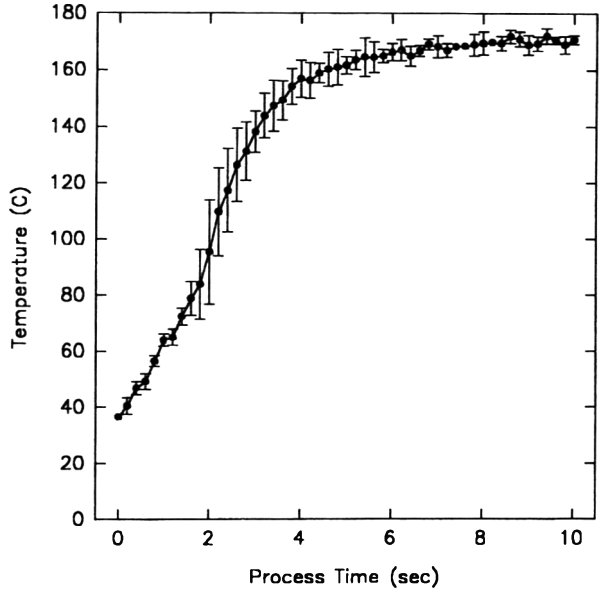

Fig. 1—Temperature rise for gluten and glutenin as related to process time. The curve represents an average of 3 experiments and is independent of moisture content and protein. Error bars represent one standard deviation.

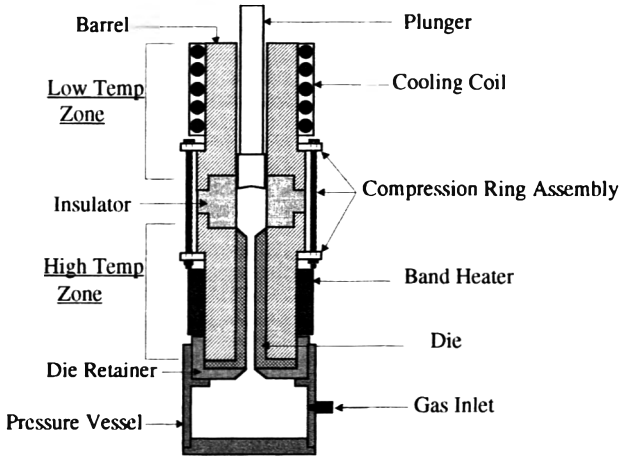

Fig. 2—Schematic of extrusion rheometer used for extrusion processing.

Model development for polymerization without shear

Soluble protein disappearance model. In Eq. (1) to (4) the reaction was followed by a change in soluble protein fraction (F_s). As polymerization proceeded, F_s decreased. This protein was not a part of the growing polymer matrix and was, therefore, soluble in denaturing solvents, such as urea and SDS. This soluble protein was not only residual monomer (single protein subunits) but also a distribution of short chain polymers (polypeptides) (Fig. 3). For modeling purposes, therefore, the soluble protein fraction characterized the change in monomer and short chain polymers with process time. The disappearance of F_s could be expressed as

$$\frac{dF_s}{dt} = -kF_s^{\beta} \tag{6}$$

where k is the apparent rate constant, and β is the reaction order. Since the process is non-isothermal the Arrhenius expression may be substituted for k to give

$$\frac{dF_s}{dt} = -Ae^{\left(\frac{-E}{RT}\right)}F_s^{\beta} \tag{7}$$

Disulfide bond formation model. The primary reaction that occurs dur-

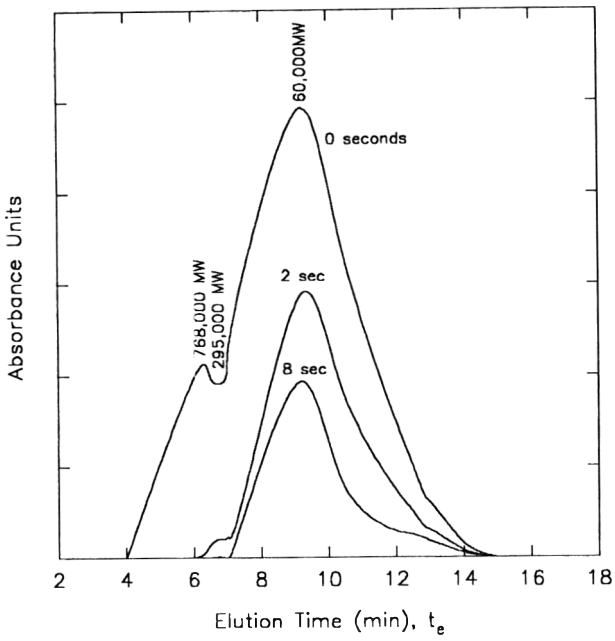

Fig. 3—Molecular weight distribution for gluten processed for different times.

ing polymerization is cross-linking through disulfide bonds (Ewart, 1968; Schofield et al., 1983; Kaczkowski and Mieleszko, 1980: Graveland et al., 1978).

$$\begin{matrix} HS{-}P{-}SH & + & HS{-}P{-}SH & \xrightarrow{k} & HS{-}P{-}S & \text{——} & S{-}P{-}SH \\ | & & | & & | & & | \\ SH & & SH & & SH & & SH \end{matrix} \quad (8)$$

SH are sulfur functional groups in the protein (P) backbone. The reaction rate would be

$$\frac{dS}{dt} = k(SH)^{\beta} \quad (9)$$

For every two thiol groups that react one disulfide bond is formed. Therefore, the difference between initial thiol concentration, SH_0, and thiol concentration at any conversion, SH, is $SH_0 - SH = 2(S - S_0)$. Defining conversion as, $X = (S - S_0)/(S_{max} - S_0)$ and $\varepsilon = S_{max} - S_0$, then $S = S_0 + X\varepsilon$, $dS/dt = \varepsilon\, dX/dt$, and $SH = SH_0 - 2X\varepsilon$. The rate expression becomes:

$$\frac{dS}{dt} = \varepsilon \frac{dX}{dt} = k(SH_0 - 2X\varepsilon)^{\beta} \quad (10)$$

Substituting $C = SH_0/2\varepsilon$ and $k_{app} = 2^{\beta} k = Ae^{(-E/RT)}$ results in the non-isothermal rate equation:

$$\frac{dX}{dt} = \varepsilon^{\beta-1} (C-X)^{\beta} Ae^{(-E/RT)} \quad (11)$$

Model development for polymerization with shear

Polymerization of nonsheared gluten proteins during thermal processing was represented by Eq. (8). However, high shear stresses break polymers down to much lower final molecular weights (Porter and Casale, 1985) particularly when the polymers cannot disentangle or bend sufficiently in response to shear. Degradation arising from breaking disulfide linkages was assumed to be the opposite of the reaction shown in Eq. (8) but not reversible since chain scissions would be random (MacRitchie, 1975; Porter and Casale, 1985)

$$\begin{matrix} HS{-}P{-}S & \text{——} & S{-}P{-}SH & \xrightarrow{k_d} & HS{-}P{-}SH & + & HS{-}P{-}SH \\ | & & | & & | & & | \\ SH & & SH & & SH & & SH \end{matrix} \quad (12)$$

The rate expression for the combined Eq. (8) and (12) is

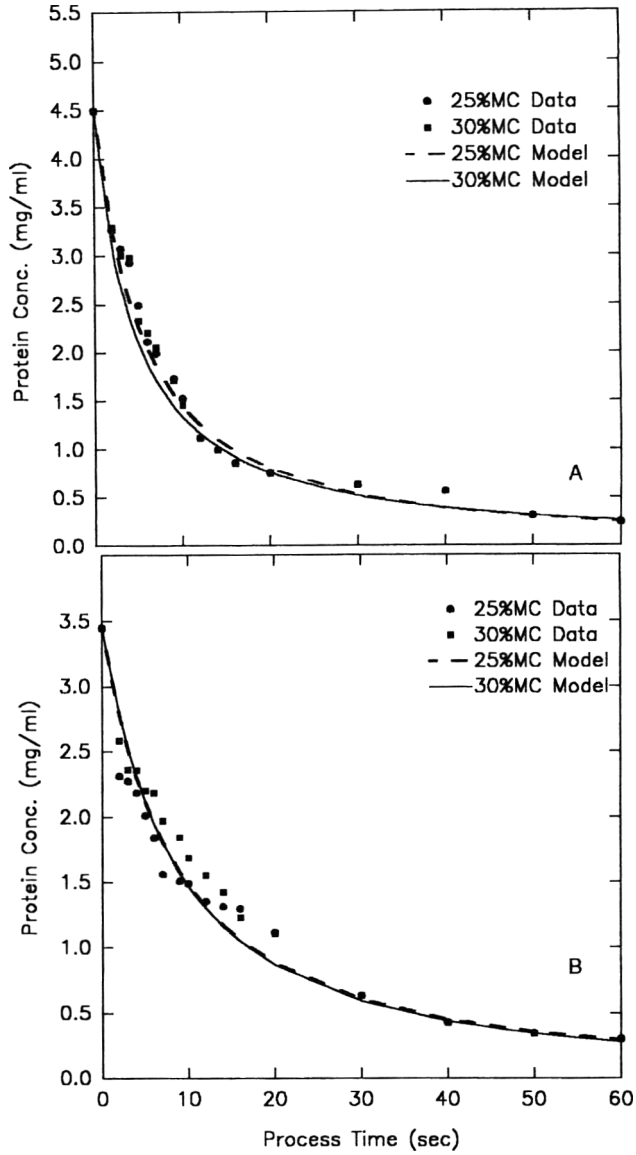

Fig. 4—Soluble protein model fit to experimental data for A: gluten, B: glutenin. Protein concentration is for soluble protein. MC moisture content.

$$\frac{dS}{dt} = k_p (SH)^{\beta} - k_d S^{\alpha} \quad (13)$$

Expressing in terms of the conversion, X, and rearranging:

$$\frac{dX}{dt} = 2^{\beta} k_p \varepsilon^{\beta-1} (C-X)^{\beta} - k_d \varepsilon^{\alpha-1} (D+X)^{\alpha} \quad (14)$$

Defining $C = SH_0/2\varepsilon$, $D = S_0/\varepsilon$, $k_{p\,apparent} = 2^{\beta}k_p = A_p e^{(-Ep/RT)}$ and $k_d = A_d e^{(-E_d/RT)}$, the nonisothermal expression for polymerization and degradation for the reaction mechanism in Eq. (8) and (12) is:

$$\frac{dX}{dt} = \varepsilon^{\beta-1} (C-X)^{\beta} A_p e^{(-E_p/RT)} - \varepsilon^{\alpha-1} (D+X)^{\alpha} A_d e^{(-E_d/RT)} \quad (15)$$

Equation (15) could be transformed to an expression dependent on shear rate by differentiating X with respect to $\dot{\gamma}$ realizing $t = 8L/D\dot{\gamma}$, where L is die length and D, die diameter.

RESULTS & DISCUSSION

Polymerization model without shear

Soluble protein disappearance model. Changes in gluten and glutenin processed according to the temperature rise curves (Fig. 1) were compared (Fig. 4). The reaction was >80% com-

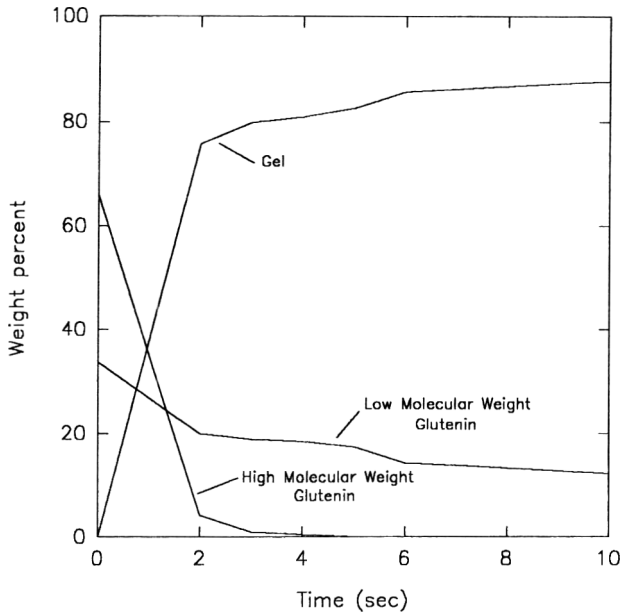

Fig. 5—Weight percents of soluble protein as related to reaction time using SEC, high MW glutenin, low MW glutenin, and gel.

Table 1—Estimated parameters for monomer disappearance model[a]

	Gluten		Glutenin	
	25%MC	30%MC	25%MC	30%MC
A $(sec^{-1})^{-\beta}$	0.57	0.85	0.62	0.92
E (kcal/mol)	1.6	1.9	1.9	2.1
β	2	2	2	2
r^2	0.98	0.98	0.97	0.95

[a] MC = moisture content, w.b.

Table 2—Estimated parameters for disulfide bond formation model[a]

	Gluten		Glutenin	
	25%MC	30%MC	25%MC	30%MC
A $(sec^{-1})(nmol/mg)^{1-\beta}$	1.0	1.0	1.6	1.9
E (kcal/mol)	0.90	1.1	0.80	1.1
β	0.6	0.7	0.3	0.5
C	1.8	1.2	3.3	4.6
r^2	0.98	0.97	0.95	0.93

[a] MC = moisture content, w.b.

plete after 10 sec during non-isothermal treatments. Gliadin is known to be heat stable due to its smaller, compact configuration (Tatham and Shewry, 1985, Schofield et al., 1983) and no appreciable reaction was observed up to 60 sec.

A numerical software, SimuSolv (Dow, Midland, MI), was used to solve equation (7) using a Runge-Kutta integration technique. SimuSolv uses a Nelder-Mead optimization scheme to fit the adjustable parameters of a model, in this case, β, A, and E. The model highly correlated ($r^2 \geq 0.95$) with experimental data. Table 1 shows optimized parameters for Eq. (7). Our activation energies fell at the lower end of the range reported for plant proteins (0.66–67 kcal/mol) (Chen et al., 1978; Mackey and Ofoli, 1990; Arce et al., 1982). Hoyer (1968) reported a protein concentration dependence on activation energy for thermal denaturation of pepsin and ovalbumin. A 4% increase in protein concentration resulted in a 70% decrease in activation energy determined by differential thermal analysis (DTA). Our data followed that trend except for a lower percentage decrease in activation energy. Our activation energy for gluten was an order of magnitude lower than reported by Pence et al. (1953). Their activation energy was based on 50% moisture gluten at 80–90°C whereas our moisture was 25–30% at 90–175°C. High MW glutenin rapidly decreases as gelation approaches a maximum (from network formation) and low MW glutenin gradually decreases as gelation proceeds (Fig. 5).

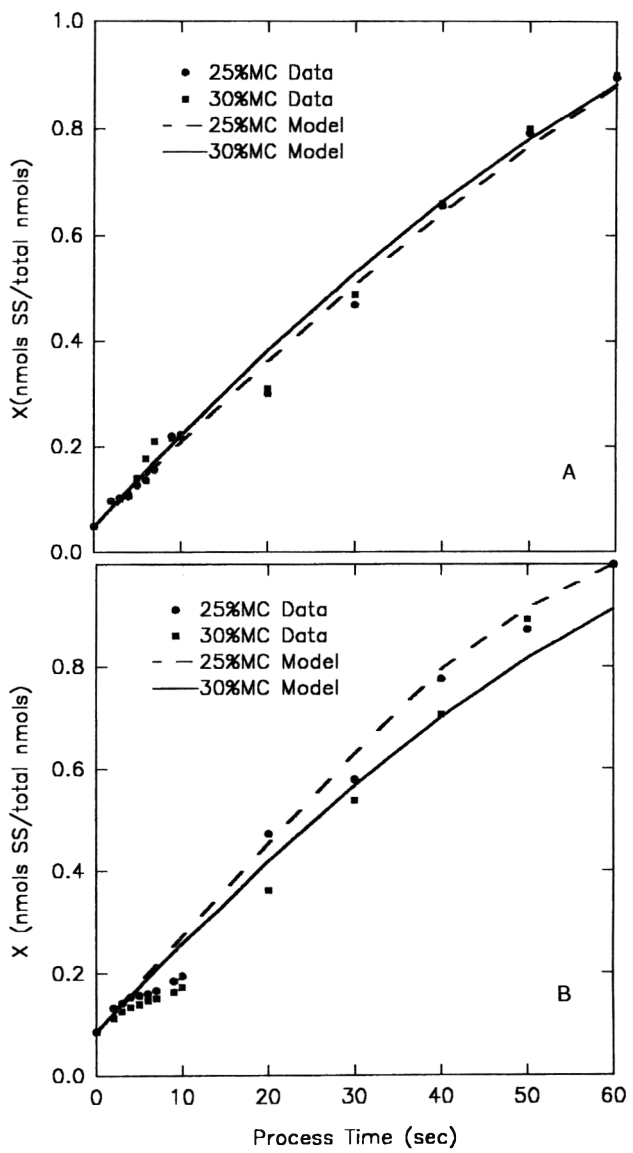

Fig. 6—Disulfide model fit to experimental data for A: gluten, B: glutenin. MC moisture content.

Table 3—Estimated parameters for overall model of polymerization (p) and degradation (d) during extrusion at extrudate temperatures of 84–160°C and shear rates of 10–275 sec^{-1}[a]

	Gluten		Glutenin	
Parameter	25%MC	30%MC	25%MC	30%MC
A_p	1.5	1.7	1.0	1.0
A_d	0.50	1.10	1.10	1.0
E_p(kcal/mol)	8.2	4.8	2.3	2.0
E_d(kcal/mol)	0.90	0.70	1.0	0.50
β	1.5	1.2	0.9	0.8
α	0.6	0.7	0.7	0.7
C	7.8	6.1	4.0	4.8
r^2	0.93	0.90	0.94	0.96

[a] MC = moisture content, w.b.

Disulfide bond formation model. Samples analyzed for soluble protein were also analyzed for disulfide bond concentration to determine changes of concentration with time. Results of model fit (Eq. 11) to the data were compared (Fig. 6). The same numerical techniques were used as before. Predicted model parameters are listed in Table 2.

The stoichiometry of thiol and disulfides in gluten is not known and the fractional reaction order would indicate non-stoichiometric reactions. The stoichiometric ratio, C, in Eq. (11) was a parameter determined in the optimization scheme of

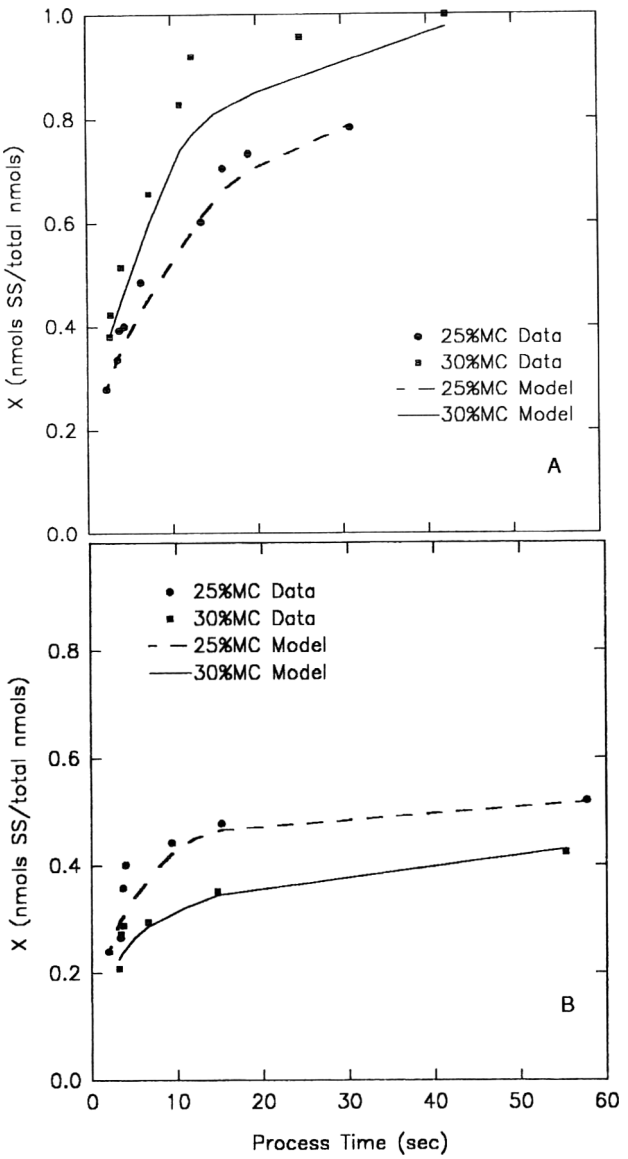

Fig. 7—Extent of polymerization, X, with shear rates for 25% and 30% moisture at 180°C for A: gluten, B: glutenin. Solid lines are model simulations.

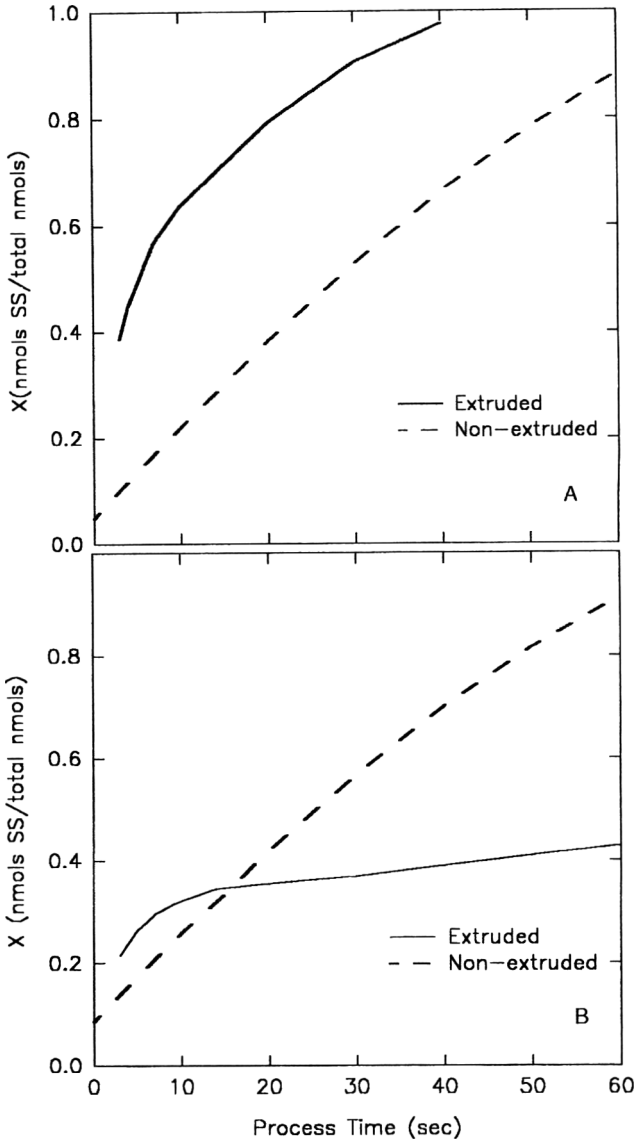

Fig. 8—Extent of reaction with process time for extruded and nonextruded A: gluten, B: glutenin samples at 30% moisture content.

SimuSolv. It was experimentally determined to be about 0.10. This agreed well with published values of 0.011–0.10 (Bloksma, 1975; Graveland et al., 1978). The value as determined by our model is an adjustable parameter.

Soluble protein data showed that 70–80% of the reaction had occurred within the first 10 sec (Fig. 4). Disulfide bond data showed that 20–25% of the reaction had occurred within the same time (Fig. 6). This indicated that with a small increase in disulfide bond formation a large increase in network formation resulted. Disulfide bonds continued to form beyond the 10 sec time period with little subsequent network formation. This substantiated the crosslinked, thermosetting characteristics of these protein polymers.

Research results (Ledward and Tester, 1994) have begun to elucidate the importance of isopeptide aggregation, free radical-initiated crosslinking, and Maillard reactions in protein polymerization during extrusion. We hypothesized that disulfide bonds were superimposed upon the network already formed by crosslinks resulting from non-disulfide reactions. Since these reactions may be important in contributing to polymerization, their exclusion is a limitation to our disulfide bond polymerization theory.

Polymerization model with shear

Using the same software the parameters, A_p, E_p, β, A_d, E_d, and α in Eq. (15) were determined (Table 3). The extent of reaction for gluten and glutenin polymers at 180°C were compared (Fig. 7). Polymerization increases with increasing process time (or with decreasing shear rate). Viscosity decreases with increasing temperature and, therefore, subsequent degradation would decrease allowing a net polymerization. This agreed with the analysis of Bueche (1960) and Casale and Porter (1971) for polymers.

Model predictions for polymerization of extruded samples were compared to nonextruded samples (Fig. 8). Polymerization of gluten may be the controlling mechanism during extrusion, since polymerization for extruded samples was higher than nonextruded samples at all process times for 30% moisture (Fig. 8a). Mechanical degradation of glutenin was the controlling mechanism during extrusion after about 12 sec of process time (Fig. 8b). Prior to 12 sec polymerization dominated. The effects observed for glutenin would be expected for a high molecular weight polymer. The explanation for gluten (Fig. 8a) is not as clear since this polymer is very complex and heterogeneous. Gliadin represents almost 40% of the gluten complex by weight. Since it is heat stable, its presence in the gluten matrix may

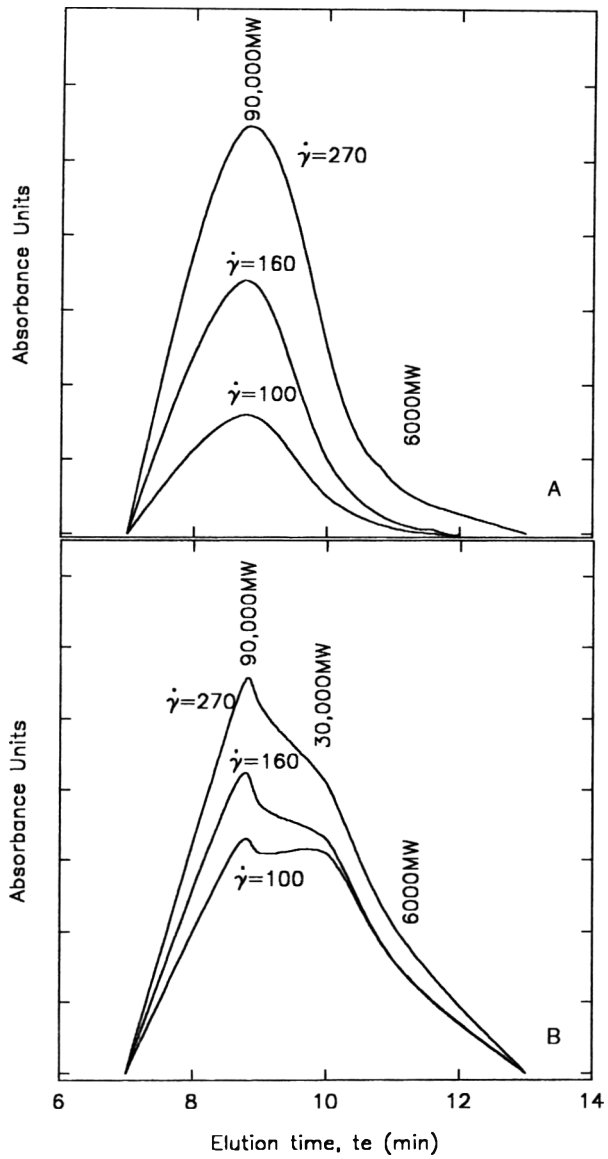

Fig. 9—Molecular weight distribution of 30% moisture A: gluten, B: glutenin extruded at different shear rates.

provide a buffer to shear forces and facilitate polymerization by reducing effects of mechanical stress. Graveland and Henderson (1987) described gliadin as a diluent that functioned to reduce the stiffness of gluten. Our results support the diluent hypothesis and indicate that gliadin acted as an inert filler. Additionally, high molecular weight molecules would align with the field of flow and thus provide more reaction sites for polymerization. The presence of gliadin ensures that these high molecular weight molecules remain intact and free from degradation effects.

By SEC (Fig. 9) glutenin exhibited a broad molecular weight distribution indicating greater degradation than observed for gluten. Degradation duc to peptide bond scission is unknown in dough systems (MacRitchie, 1975). Peptide bonds have higher bond energies than disulfide bonds and thus, disulfide bonds would break first. Weaker non-covalent bonds were not investigated in our analysis. Hydrophobic interactions, ionic, and hydrogen bonds might be important, but to a lesser degree.

Future research will need to address the diffusional dependence of the rate constant. This diffusional dependence is related to segmental diffusion and not mass transfer. Soh and Sunberg (1982) have shown the impact of segmental diffusion on the rate constant for addition polymerization. Chain mobility decreases with conversion. As polymer molecular weight increases, entanglements and branching reduce the distance a chain segment can diffuse through the polymer network to react with another segment. The higher the molecular weight, the lower the probability that a given chain segment would react with another segment because of reduced mobility. Such reduced chain mobility would effectively reduce the rate of reaction.

CONCLUSIONS

THE REACTION MECHANISM in gluten during extrusion processing was dominated by polymerization with gliadin providing a buffer to shear forces. Disulfide bond analysis showed that gliadin did not participate in polymerization reactions for residence times evaluated. Realizing that Maillard reactions and free-radicals could also produce crosslinkages, this research has provided insight into the extent disulfide bonds contribute to polymerization reactions and the rate of such reactions. The three kinetic models of monomer disappearance, disulfide bond formation and combined polymerization and degradation during extrusion should be useful in extrusion process modeling.

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Folic Acid Content in Thermostabilized and Freeze-Dried Space Shuttle Foods

H.W. LANE, J.L. NILLEN, and V.L. KLOERIS

ABSTRACT

This study was designed to determine whether freeze-dried and thermostabilized foods on a space shuttle contain adequate folate and to investigate any effects of freeze-drying on folacin. Frozen vegetables were analyzed after three stages of processing: thawed; cooked; and rehydrated. Thermostabilized items were analyzed as supplied with no further processing. Measurable folate decreased in some freeze-dried vegetables and increased in others. Folacin content of thermostabilized food items was comparable with published values. We concluded that although the folacin content of some freeze-dried foods was low, adequate folate is available from the shuttle menu to meet RDA guidelines.

Key Words: broccoli, green beans, cauliflower, freeze-drying, folacin

INTRODUCTION

FOLIC ACID (folacin) is a water-soluble, B-complex vitamin found primarily in organ meats, whole grains, and green leafy vegetables. In humans, folacin acts as a coenzyme in the transport of single carbon fragments during amino acid metabolism and nucleic acid synthesis. Daily requirements depend on metabolic and cell turnover rates (Herbert and Colman, 1988). The estimated safe and adequate daily dietary intakes are for adult men 200 mg and women 180 mg (NRC, 1989). Folacin deficiencies lead to impaired cell division and megaloblastic anemia (Nelson and Davey. 1991). Enterohepatic circulation tends to conserve the body pool of folate (Steinberg, 1984) and regular consumption of the recommended amount should maintain adequate body stores to prevent depletion during brief periods of reduced intake.

The amount of folate available from processed foods varies depending on the preparation method. Klein et al. (1979) determined that both water-to-vegetable ratio and length of cooking time affected the magnitude of folacin loss when cooking vegetables. Hurdle et al. (1968) found decreases in folacin content of up to 90% in boiled vegetables such as cabbage, potatoes, and broccoli and reductions caused by frying were more variable. Bread dough reportedly lost about one-third of the folacin during baking (Butterfield and Calloway, 1972) but toasting did not cause additional loss. Santini et al. (1962) measured folate in homogenates of whole meals and reported losses from cooking ranging from 0 to 45%. Very little information is available concerning the effect of freeze-drying on folacin content. Clifford et al. (1991) analyzed several cooked vegetables that had been freeze-dried and reported folate levels comparable to fresh vegetables. However, Karmas et al. (1962) and Goldblith and Tannenbaum (1966) reported losses of other sensitive vitamins such as thiamine and ascorbic acid after freeze drying.

The diet provided for space shuttle astronauts primarily consists of freeze-dried or thermostabilized products with few fresh foods. Thermostabilized foods are warmed during flight, and freeze-dried products are rehydrated with hot water (68–74°C). As the duration of space flight is extended, inadequate intake of labile vitamins, including folate, may pose health risks to the crews. To determine whether such space foods contain adequate folate, our objective was to quantify folic acid in a variety of shuttle menu items as served in flight. We also investigated the effect of freeze-drying on folacin content of a variety of vegetables.

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Table 1—Percent moisture in vegetables after three stages of processing*

Vegetable	Processing method: Frozen, thawed %	Frozen, cooked %	Cooked, freeze-dried, rehydrated %
Asparagus	90.5 ± 0.3[a]	88.8 ± 1.0[b]	87.5 ± 0.8[c]
Broccoli & Cheese	84.1 ± 2.2[a]	87.1 ± 1.1[b]	73.8 ± 1.3[c]
Corn, Broccoli, & Pasta	79.2 ± 1.0[a]	80.6 ± 0.4[b]	76.9 ± 1.5[c]
Cauliflower & Cheese	88.9 ± 0.5[a]	87.7 ± 0.9[b]	84.5 ± 0.7[c]
Creamed Spinach	80.4 ± 0.4[a]	81.1 ± 0.8[a]	78.5 ± 1.5[b]
Green Beans & Broccoli	86.2 ± 0.6[a]	85.0 ± 2.4[a]	78.9 ± 2.6[b]
Green Beans with Mushrooms	89.9 ± 0.2[a]	90.0 ± 0.5[a]	84.7 ± 0.4[b]
Mixed Italian Vegetables	86.0 ± 1.0[a]	81.9 ± 1.1[b]	73.1 ± 0.9[c]

* Data are mean ± SD per gram dry weight of ten replicates of each sample
[a,b,c] Different superscripts in the same row denote statistically significant differences at $p < 0.05$.

MATERIALS & METHODS

Processed food samples

Freeze-dried shuttle foods were prepared according to food-processing standards established by the NASA Flight Crew Support Division. Frozen vegetables were cooked according to package directions before freeze-drying. An exception was green beans, which were thawed in a steam-jacketed kettle instead of cooked and then mixed with canned mushrooms at a ratio of 18% mushrooms to 82% green beans (Bourland, 1989). For freeze-drying, a Stokes Model 24P freeze-drier (Pennsalt Chemical Corp., Philadelphia, PA) was chilled to −34 ± 12°C and then vegetables were placed on shelves to a depth of <4 cm. The vegetables were frozen and the freeze-drier activated. When the vacuum reached 100 microns, shelves were heated not to exceed 26°C during drying. Product temperatures were maintained between 21–27°C for 8 hours before food was removed from the freeze-drier and vacuum-packed for storage (Bourland, 1992).

Commercially available frozen vegetables identical to the rehydratable shuttle foods were purchased from local retailers and thawed or cooked according to package directions before analysis. The thermostabilized items analyzed for this study were either commercially available canned products or U.S. Military ready-to-eat meals (MRE) contained in foil pouches. Such foods are stowed "as is" on the Space Shuttle, in original containers, with no further processing.

Folacin extraction and deconjugation

Extractions were carried out in quintuplicate under gaseous nitrogen. A 10 g aliquot of each food sample was mixed with 90 mL of 50 mM HEPES/CHES extraction buffer, pH 7.85 (Wilson and Horne, 1984), and heated at 100°C for 10 min. Samples were cooled to room temperature (≈23°C) and homogenized using an Omni Mixer Homogenizer (Omni International, Inc., Waterbury, CT). Duplicate aliquots from each homogenate were digested with a modified tri-enzyme procedure (Martin et al., 1990). Briefly, homogenate was adjusted to pH 7.0 with either sodium hydroxide or hydrochloric acid, then 20 mg α-amylase and 3 mg

Table 2—Folate content of vegetables after three stages of processing*

Vegetable	Frozen, thawed folate µg/g	Frozen, cooked		Cooked, freeze-dried, rehydrated	
		Folate µg/g	% Retention	Folate µg/g	% Retention
Asparagus	39.800 ± 1.770[a]	27.911 ± 2.500[b]	70.1	11.576 ± 1.389[c]	29.1
Broccoli & Cheese	3.522 ± 0.314	3.702 ± 0.216	105.1	3.366 ± 0.413	95.6
Corn, Broccoli, & Pasta	2.740 ± 0.354[a]	2.404 ± 0.329[a]	87.7	3.530 ± 0.431[b]	128.8
Cauliflower & Cheese	10.297 ± 1.138[a]	10.564 ± 1.163[a]	102.6	5.029 ± 0.440[b]	48.8
Creamed Spinach	5.019 ± 0.276[a]	5.005 ± 0.249[a]	99.7	8.019 ± 0.696[b]	159.8
Green Beans & Broccoli	5.893 ± 0.507[a]	5.516 ± 0.654[a]	93.6	2.415 ± 0.266[b]	41.0
Green Beans with Mushrooms	4.107 ± 0.352[a]	3.312 ± 0.359[b]	80.6	4.272 ± 0.261[a]	104.0
Mixed Italian Vegetables	6.126 ± 0.498[a]	6.573 ± 0.465[b]	107.3	3.915 ± 0.189[c]	63.9

* Data are mean ± SD per gram dry weight of ten replicates of each sample.
[a,b,c] Different superscripts in the same row denote statistically significant differences at $p < 0.05$.

Table 3—Folate content of representative foods supplied aboard a space shuttle*

Food	Type of processing	Folate µg/g
Chicken Salad Spread	Commercially canned	0.341 ± 0.044
Ham Salad Spread	Commercially canned	0.293 ± 0.039
Tuna Salad Spread	Commercially canned	0.272 ± 0.031
Meatballs w/Spicy Tomato Sauce	Foil Pouches	0.437 ± 0.025
Scrambled Eggs	Freeze-dried	1.427 ± 0.151
Mexican Scrambled Eggs	Freeze-dried	1.673 ± 0.174
Tomatoes and Eggplant	Foil Pouches	1.588 ± 0.121
Raspberry Yogurt	Commercially canned	0.250 ± 0.025
Chocolate Pudding	Commercially canned	0.250 ± 0.023
Instant Orange Drink	Commercial powder	4.717 ± 0.462
Freeze Dried Orange Juice Crystals	Freeze-dried	2.258 ± 0.412

* Data are mean ± SD per gram dry weight of ten replicates of each sample.

of chicken pancreas conjugase (Kirsch and Chen, 1984) were added/g food. The homogenates were incubated at 37°C for 4 hr, then 2 mg of protease/g food were added to the extract. Samples were incubated overnight at 37°C and then heated at 100°C for 5 min to deactivate enzymes. After centrifugation at 20,000 × *g* for 20 min, the supernatant was filtered through a 0.45 µm filter and either analyzed immediately or frozen at −20°C for no more than 1 wk.

Folacin assay

Folinic acid (Sigma Chemical Co., St. Louis, MO) standard was prepared from a racemic mixture to provide a solution equivalent to 2 ng/mL of the 6S folinic acid isomer. Food extracts were diluted in assay buffer (0.1M phosphate buffer, pH 6.3, containing 1 mg/mL ascorbic acid) to about 0.2 ng folic acid/100 mL. Folacin content was measured in duplicate using a 96-well microplate modification of the microbiological procedure described by Tamura (1990) using *Lactobacillus casei* (ATCC 7469, American Type Culture Collection, Rockville, MD). Standards (150 µL) and samples (200 µL) were pipetted into appropriate wells of the microtiter plate (Falcon 3072, Curtin Matheson Scientific Inc., Houston, TX) and well volumes were adjusted to 300 µL with assay buffer. Six serial dilutions (ranging from 1 to 32 times dilution) of standards and samples were made using a multichannel pipettor. Double-strength folic acid casei medium (150 µL; Difco Laboratories, Detroit, MI) was then added to each well. Cryoprotected *L. casei* (Wilson and Horne, 1982) was diluted 1:9 with 0.9% sodium chloride, and 20 µL aliquots of the suspension were pipetted into all wells. Microplates were covered and incubated for 18 hr at 37°C. After incubation, the contents of each well were mixed and bacterial growth determined by measuring turbidity with a microplate spectrometer (Emax Precision Microplate reader, Molecular Devices, Menlo Park, CA). The concentration of folic acid (ng/well) was calculated by comparing absorbance readings of unknowns with those from diluted standards. Final concentrations were reported in µg/g dry weight of food by multiplying by appropriate dilution factors and correcting for moisture content.

Reproducibility was monitored by analyzing two concentrations of commercially prepared control sera (Immunoassay Control Serum, Bio-Rad, Anaheim, CA) each time an assay was performed. Coefficients of variation for control sera at the low (mean 3.9 ng/mL) and high (mean 13.8 ng/mL) concentrations were 11.8% and 11.4%, respectively. These values compared favorably with those reported by Tamura (1990). Post hoc analysis of the 24 standard curves used throughout the study revealed a mean R^2 of 0.97 ± 0.03 (± SD).

Moisture analysis

Thermostabilized, rehydrated, thawed, and cooked food items were prepared as described above, and aliquots were measured for moisture content at the time of folacin extraction. Samples (10g each) were weighed into crucibles and desiccated in a vacuum oven under 730 mm Hg for 15 hr at 70°C. After removal from the oven they were covered, cooled in a desiccator, and re-weighed.

Data analysis

Ten data points for each food item were obtained by extracting five different samples and performing the digestion on duplicate aliquots from each extraction. These procedures were carried out over several days to account for any day-to-day variability. In order to minimize the effects of moisture on analysis of folacin, all results were computed/g dry weight. Percent folate retention (%R) was calculated with the following formula:

$$\%R = \frac{\mu g \text{ Folate} \times g \text{ cooked food (dry wt)}^{-1}}{\mu g \text{ Folate} \times g \text{ frozen food (dry wt)}^{-1}} \times 100$$

Data were analyzed by analysis of variance using SAS/STAT for the PC, Version 6.03 (SAS Institute Inc., Cary, NC). If a difference was detected at the $p < 0.05$ level, Duncan's multiple range test was used to determine differences between individual processing methods.

RESULTS & DISCUSSION

PERCENT MOISTURE IN THAWED, cooked, and rehydrated foods (Table 1) showed, as expected, cooking tended to decrease moisture. Decreases in moisture content of rehydrated foods can be attributed to incomplete rehydration and loss of water-holding capacity. King et al. (1968) reported that several conditions during freeze-drying, such as outer-surface and frozen-zone temperatures of food during processing and residual moisture content after processing, altered rehydration ratio. Karel (1963) attributed the loss of water-holding capacity of freeze-dried plant foods to increases in crystallinity of cellulose and starch.

The mean folacin content and percent folacin retention of thawed, cooked, and rehydrated foods were compared (Table 2). Although most foods analyzed were mixtures, mean folate values were similar to those reported by others for each primary vegetable. Martin et al. (1990) found folate levels of 1.30 µg/g wet weight for frozen cauliflower and 1.26 µg/g for frozen spinach. In their review, Perloff and Butrum (1977) found folacin levels (per gram wet weight) of 0.56 µg in cooked broccoli and 0.44 µg in raw green beans. Comparable wet-weight folacin from our study were 1.14 µg/g (frozen cauliflower and cheese), 0.98 µg/g (frozen creamed spinach), 0.48 µg/g (cooked broccoli and cheese), and 0.42 µg/g (frozen green beans with mushrooms).

As expected, freeze-drying affected folacin concentration in all foods except broccoli with cheese and green beans with

mushrooms. Folate was lost inconsistently during freeze-drying and decreases were observed for asparagus, cauliflower with cheese, green beans with broccoli, and mixed Italian vegetables. These losses probably resulted from oxidation reactions. Lipids are oxidized in dehydrated foods (King, 1970); moreover, the increase in internal surface area increases such oxidative reactions in freeze-dried foods, and thus would decrease amounts of other vitamins sensitive to oxidation (Karel, 1963). Karmas et al. (1962) attributed the loss of thiamine in dehydrated pork to oxidation caused by replacement of tissue water with air. Although foods provided for space shuttle crews are dried in a reduced oxygen environment, folacin is oxidized easily and may be converted during processing and storage to forms that are not as readily utilized by *L. casei* (Phillips and Wright, 1982; Ruddick et al., 1978).

Apparent increases in folate concentration in freeze-dried corn, broccoli, and pasta and creamed spinach (Table 2) may be accounted for by one or more of the following: Folate present in both foods may have bound to food residues during extraction. Santini et al. (1962) and Gregory et al. (1990) reported incomplete extraction of folacin from some foods, which they attributed to interaction of folate with insoluble residues or retention by physical entrapment. Tamura and Stokstad (1973) indicated that the bioavailability of folate in foods may be affected by the amount of folate-binding protein present. Another possibility could be destruction of folate-conjugase inhibitors during freeze-drying. Krumdieck et al. (1973) noted the presence of a conjugase inhibitor in several varieties of beans. That was supported by Bhandari and Gregory (1990), who found that many foods (including spinach) contained substances that inhibit conjugase activity. Since freeze-drying foods reduces ATPase activity and denatures some meat proteins (Hamm, 1960), other enzymes and proteins may also be affected. Finally, freeze-drying of some foods may convert folacin to forms that are used more efficiently by *L. casei*. Both Ruddick et al. (1978) and Phillips and Wright (1982) reported that the growth response of *L. casei* to N^5-methyl-tetrahydrofolic acid was low when compared with pteroylglutamic acid and N^5-formyl-tetrahydrofolic acid.

The folacin values from a variety of commercially prepared shuttle menu items (Table 3) compared favorably with published data. Martin et al. (1990) found 0.095 μg of folate/g (wet weight) of canned tuna; we found 0.084 ± 0.009 μg folate/g (wet weight) of the tuna-salad spread. In contrast, others have reported folate concentrations (per gram wet weight) of 0.11 μg/g for yogurt, 0.11 μg/g for smoked ham, and 0.49 μg/g for hard-cooked eggs (Perloff and Butrum, 1977). Folacin values for similar shuttle foods (per gram wet weight) were 0.08 μg/g for canned yogurt, 0.09 μg/g for ham salad, and 0.42 μg/g for rehydrated scrambled eggs. Folacin in other shuttle foods compared favorably with values derived from the USDA Nutrient Database (USDA, 1993).

Effects of thermostabilization on folacin content of preprocessed shuttle foods could not be assessed because thermostabilized shuttle foods are provided in that form from commercial or military vendors. Folate was lost through the cooking process (Table 2) and further losses through thermostabilization would probably be minimized by the closed-system process. This type of processing theoretically reduces oxidation reactions, which have been implicated in destruction of folate during freeze drying (King, 1970; Karel, 1963; Karmas et al., 1962).

CONCLUSIONS

ALTHOUGH THE FOLACIN CONTENT of some freeze-dried foods is low, adequate amounts are available on the shuttle menu to meet RDA guidelines. The effect of space flight on folate bioavailability remains to be determined and may alter folate requirements. Diet counseling will be necessary to ensure that each crewmember understands the importance of consuming adequate folacin and knows which foods contain this vitamin so that appropriate foods are consumed. Folacin contents of the remaining food items also need to be analyzed.

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Ascorbic Acid and 5-Methyltetrahydrofolate Losses in Vegetables with Cook/Chill or Cook/Hot-Hold Foodservice Systems

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ABSTRACT

The percent retention of vitamin C and 5-methyltetrahydrofolate in seven cooked vegetables was analyzed using HPLC and compared in simulated cook/chill vs cook/hot-hold hospital foodservices. Retention of vitamins after conduction and infra-red reheating was investigated. Vegetables reheated after one day of chilled storage had greater losses of both vitamins compared to those held at 72°C for 30 min, but better vitamin retention than vegetables held hot 2 hr. There was no significant difference in nutrient retention due to method of reheating. If warm-holding is restricted to < 90 min, vitamin retention in vegetables is likely to be higher in a cook/hot-hold foodservice than with a cook/chill system.

Key Words: ascorbic acid, methyltetrahydrofolate, foodservice, cook-chill

INTRODUCTION

KNOWLEDGE OF NUTRIENT CONTENTS of food served to patients is needed to ensure the nutritional adequacy of hospital menus and for planning special diet modifications. Tables of food composition are commonly used to determine nutrient contents of food served to patients. There are usually notable discrepancies between published values and the actual nutritional content of food at the point of service in institutional food service settings (Lachance and Fisher, 1988). Some attempts have been made to quantify likely losses during cooking and service (Lachance, 1975; Rumm-Kreuter and Demmel, 1990). Few data are available on nutritional effects of many new technological developments in foodservice industries (Livingston et al., 1973).

There has been a trend to replace cook/hot-hold foodservices with cook/chill systems where food is cooked, chilled and held up to 5 days before reheating and service to patients. Such systems allow centralized production units to replace kitchens in individual hospitals and may provide substantial cost savings. The limited work that has been done on nutritional effects of cook/chill foodservices has concentrated on relatively few nutrients (principally ascorbic acid, thiamin and riboflavin). It has involved a restricted range of foods, and has not evaluated newer methods of reheating, such as conduction reheating (Aladdin Industries).

In Australia most hospital cook/chill foodservices use blast chilling with storage of food for up to 3 days and very few produce extended shelf life products using Capkold or similar systems. No national standards exist for the time/temperature holding of food in hospitals. Some local recommendations have been made (NSW Department of Health, 1989; Williams and Brand Miller, 1992), but most institutions follow the British standards for cook/chill systems (Department of Health, 1989).

Our objective was to examine losses of vitamin C and folate in seven common vegetables, in simulated cook/hot-hold or cook/chill foodservices, using 3 different reheating methods. These two vitamins were chosen for study because vitamin C and folate are among the seven critical vitamins suggested for further research in evaluation of food processing by the COST 91-Sub-group 4-Nutrition (Pietrzik, 1984). They are both known to be highly labile, with losses of up to 80% of folate and 70% of vitamin C during cooking (Kwiatowska et al., 1989). Little data are published on losses of folate caused by chilling, storage or reheating in large-scale cook/chill catering. One study reported changes during chilled storage and microwave reheating of potatoes (Augustin et al., 1980) and another measured losses in Brussels sprouts during chilled storage (Bender, 1985).

Only vegetables were examined in our study. Vegetables are the major dietary source of folic acid (Spring et al., 1979) and can be important sources of vitamin C, contributing up to 33% of ascorbic acid intake of institutionalized patients (Löwik et al., 1993). Instead of measuring total folate in the vegetables using the AOAC microbiological method, only 5-methyltetrahydrofolate (5MeTHF) was assayed using high-performance liquid chromatography (HPLC). 5MeTHF is reported to be the predominant form of folate in most vegetables, accounting for up to 90% of biologically active forms (Chan et al., 1973; Scott and Weir, 1976; Stokstad et al., 1977; Cossins, 1984), and it has a significantly higher bioavailability than tetrahydrofolate or formyl folates when measured by 2 hr plasma levels after oral administration to humans (Brown et al., 1973). Furthermore, assay by HPLC is relatively easy and fast, and more reproducible than a microbiological assay, which can take up to 3 days/sample. By examining identical batches of food processed in either cook/chill or cook/hot-hold foodservices, our objective was to provide a direct comparison of nutrient losses caused by these two foodservice systems.

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MATERIALS & METHODS

Food preparation and sampling

Simulation of cook/hot-hold and cook/chill foodservices was used to control time and temperature of processing and selection of equipment. Three different methods of reheating chilled vegetables were examined: (1) infra-red (IR) reheating of plated individual meals, (2) IR reheating of bulk trays of chilled food, and (3) conduction reheating of plated meals.

The cooking and chilling equipment in a cook/chill production kitchen which produces about 3000 meals/day for 14 nursing homes throughout Sydney was used. For IR reheating of bulk and plated food, vegetables were cooked, rapidly chilled, stored and reheated in a Regethermic oven (Table 1). Conduction reheating, using the Aladdin plate heating system, was carried out at a demonstration kitchen of Aladdin Industries, ~ 20 min travel time from the production kitchen. Seven different vegetables were used, to include those commonly found on hospital menus and to represent a variety of shapes, sizes and textures: steamed potato, mashed potato, broccoli, peas, chopped silverbeet, pumpkin, and diced carrots.

Vegetables were procured in forms commonly used by institutional caterers: peeled fresh potatoes and pumpkin, fresh chopped silverbeet, frozen carrots, broccoli and peas. Vegetables were prepared and cooked using the most widely used methods found in a survey of 30 New South Wales (NSW) hospital foodservices (Williams and Brand Miller, 1993). Vegetables were either steamed in full-size perforated stainless steel gastronorm pans in a Zanussi FCV/E10 convection steamer (Zanussi, Via C. Battisti 12, Conegliano, Italy) or boiled in unsalted tap water in a 50 L steam-jacketed kettle (Cleveland Model FTE-40; Cleveland, 1333 E 179th St., Cleveland, OH), until judged to be just done.

Three replicate batches of each vegetable were prepared from a single lot of each original food product, as recommended by Carlson and Ta-

Table 1—Equipment used to reheat food in the cook/chill systems

System	Oven	Container	Cover
Infra-red plated	Model 415TR1 6kW oven. Regethermic International	9" China plates Regethermic	9" stainless steel Regethermic
Infra-red bulk	Model 415TR1 6kW oven. Regethermic International, 196A Miller St., North Sydney, NSW, Australia.	Stainless steel pan 530×325×50mm Regethermic	Stainless steel Regethermic
Conduction plated	Temp-Rite II Excell Rethermalization Refrigerator. Aladdin Industries Pty Ltd, 43-45 Bridge Road, Stanmore, NSW, Australia.	8" China plates Aladdin	8" insulated plastic Aladdin

bacchi (1988), with exception of carrots. Only 2 replications of diced carrots were prepared because the availability of the kitchen was limited. After cooking, each 10 kg batch of vegetables was divided and placed into four half-size (1.5 kg) and one full-size (3 kg) stainless steel gastronorm trays. These five samples were then processed (Fig 1) to simulate handling in cook/hot-hold and cook/chill hospital foodservices. Duplicate samples were taken for each vitamin analysis at the 10 stages in the simulated foodservice systems.

Cooking methods

Steamed potatoes. Ingredients were peeled whole potatoes supplied by South Pacific Salads, Smithfield, in 10 kg bags, with sodium metabisulphite added as a preservative. 10 kg of potatoes were distributed between 3 trays and steamed for 50 min.

Mashed potatoes. Potatoes were cooked as for steamed potatoes and then whipped with 3 L of warmed fresh whole milk for 3 min in a 60 L Hobart mixer (Model H600T; Hobart Manufacturing Co., Troy, Ohio) using a wire whisk.

Peas. Ingredients were frozen peas (supplied by Wattie Frozen Foods) in 2 kg packs. 10 kg of frozen peas were added to 30 L of unsalted boiling water in the kettle and cooked for 5 min after the water returned to boiling.

Broccoli. Ingredients were frozen broccoli florets (supplied by McCain Foods Aust. Pty. Ltd.) in 2 kg packs. 10 kg of frozen broccoli were added to 30 L of unsalted boiling water and cooked 5 min after the water returned to boiling.

Silverbeet. Silverbeet was purchased as a mixture of fresh chopped silverbeet leaf and stem (~ 30%) in 5 kg bags from South Pacific Salads, Smithfield. 10 kg were added to 30 L of boiling unsalted water and cooked 20 min after the water returned to boiling.

Pumpkin. Ingredients were peeled pumpkin pieces (~ 100 g each) supplied by South Pacific Salads, Smithfield in 10 kg bags. 10 kg of pumpkin were steamed in 3 trays for 50 min.

Carrots. Ingredients were frozen diced carrots (supplied by Edgell-Birds Eye) in 2 kg packs. 10 kg of frozen carrots were added to 30 L of boiling water in the kettle and cooked for 4 min after the water returned to boiling.

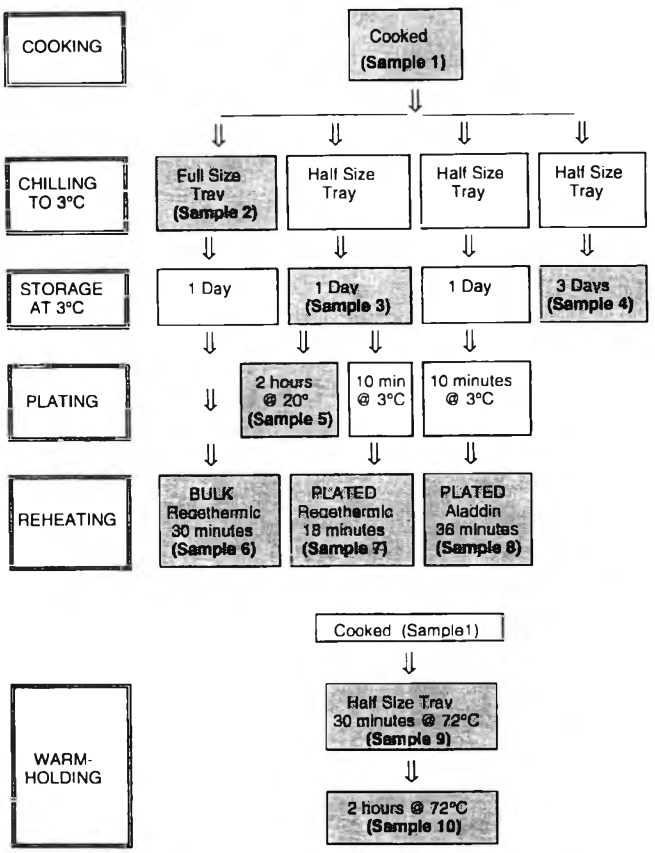

Fig. 1—Product flow diagram and experimental design for nutrient assays. Stages at which food was sampled are shaded and numbered 1–10. Three replicated experiments were conducted and duplicate samples taken at each stage for analysis.

Chilling and storage

Cooked vegetables were rapidly chilled to 3°C by placing them in uncovered 5 cm deep stainless steel trays in an air cooled Foster blast chiller (Model BQC 130; Foster Refrigeration, Oldmeadow Road, King's Lyn, Norfolk, UK) for 90 min. After chilling, vegetables were covered and stored in a 0–3°C coolroom for 1 to 3 days before reheating.

Plating

Those vegetables that were to be reheated as individual samples were served as part of a standardized plated chilled meal in order to simulate thermal conditions that would be experienced during reheating of normal whole meals. In each case the plated meal consisted of three cooked meat hamburgers (weighing 100g total), two scoops of mashed potato (120g) plus the vegetable under investigation. Serving sizes were 90g for potatoes and 60g for all other vegetables. Vegetables were served on round ceramic plates used with the 2 systems. The test vegetable was always placed in the center of the plate. Plating took place in air-conditioned rooms over 10 min and meal reheating commenced within 30 min of plating. The chilled food to be reheated in the conduction system was transported in bulk in insulated containers to the demonstration kitchen and plated there before reheating. For all food that was to be reheated, food temperatures remained <5°C throughout transport and plating before reheating.

A separate study was conducted to examine the impact of exposure of chilled food to temperatures >3°C (that had been reported during meal plating in many hospital chilled foodservices by Williams and Brand Miller, 1992). Three samples of food that had been stored for 1 day at 3°C were plated as described and then held at room temperature (20°C) for 2 hr, before being sampled for analysis (Fig 1; Sample 5).

Infra-red reheating

IR reheating was completed using the Regethermic system (Table 1). The oven contained five racks with heating elements above and below each rack. Bulk reheating of vegetables took place in one lidded full-size gastronorm stainless steel tray holding 3 kg of food placed on the middle rack, at a setting of 210°C for 30 min. As recommended by manufacturer, one cup of water was added to carrots, peas and spinach before reheating. Plated meals were covered with stainless steel lids and 9 replicate meals heated together at 190°C for 18 min, with 3 plates on each of the top, middle and bottom racks.

Conduction reheating

Conduction reheating was completed using the Aladdin system (Table 1). Plated meals were covered with a plastic insulating lid and reheated in a refrigerated cabinet by energy transfer from individually controlled heating pods under each plate. Nine plated meals were heated together and total reheating time was 36 min.

Table 2—Temperature of vegetables at time of sampling

Sample	Temp (°C) Mean (SD)
1-Cooked	82.5 (10.4)
2-Chilled	1.0 (0.8)
3-1 day storage	2.2 (1.0)
4-3 day storage	1.8 (0.6)
5-Room temp for 2 hours	15.5 (1.1)
6-IR bulk reheat	75.4 (6.5)
7-IR plated reheat	76.4 (8.7)
8-Conduction reheat	73.6 (3.6)
9-Warm-hold 30 minutes	72.1 (4.3)
10-Warm-hold 2 hours	72.2 (5.5)

Table 3—Summary of vitamin retention in all seven vegetables combined

Sample	Percent vitamin retention Mean (SEM) Vitamin C	5MeTHF
Cooked	100 (0.0)	100 (0.0)
Chilled	72.0 (4.0)	83.0 (3.3)
1 day chilled storage	66.0 (4.7)	81.8 (2.4)
3 days chilled storage	49.8 (4.9)	69.3 (4.8)
Room temp for 2 hr	62.1 (5.1)	78.4 (3.2)
Infra-red plated reheat	54.4 (4.4)	77.7 (3.5)
Infra-red bulk reheat	49.2 (5.1)	69.5 (3.6)
Conduction reheat	46.7 (4.7)	74.9 (3.7)
Warm-hold 30 min	64.5 (4.2)[a]	81.1 (3.3)[c]
Warm-hold 2 hr	40.3 (4.4)[b]	67.7 (3.9)[c]

[a] Significantly more than reheated chilled food: $p < 0.01$
[b] Significantly less than reheated chilled food: $p < 0.05$
[c] Not significantly different from reheated chilled food

Warm-holding

The warm-holding of food in cook/hot-hold food service systems was simulated by holding 1.5 kg samples of the vegetables in stainless steel half-size trays in a thermostatically-controlled warming oven up to 2 hr. The containers were covered with sealing lids to prevent excessive moisture loss. The temperature of the food was monitored continuously as described (Williams and Brand Miller, 1992) and was maintained at 72°C within a 4°C range over the 2 hrs of warm-holding.

Sampling

The total vitamin C and 5MeTHF content of the vegetables was determined at several points in the food product flow (Fig. 1). At each stage, duplicate, accurately weighed, 25g food samples were taken for each vitamin analysis. Care was taken to ensure that composite samples were collected (with a sample of the vegetable from each of the 3 reheated meals or from at least 3 different parts of the reheated bulk food containers, including the center, middle of the long side of the tray, and half way between the center and one corner) and that there was no contamination from other foods of the plated meals.

Samples for vitamin C analysis were stabilized in 25 mL of 25% metaphosphoric acid (MPA); samples for folate analysis were stabilized in 25 mL of 2% ascorbic acid, as recommended (Brubacher et al., 1985). Samples were collected in 70 mL screw-cap polycarbonate specimen jars and snap frozen immediately in dry ice before transportation in insulated containers to the laboratory for storage. Samples for vitamin C analysis were stored at −18°C for 21–80 days before preparation for analysis. Folate samples were stored at −18°C for 6 mo before preparation and analysis. All samples in each replicate batch of vegetables were prepared for analysis on the same day so that when comparing nutrient retention at different stages of the food service process, times of storage at −18°C were essentially identical for all samples being compared.

Vitamin analyses

Vitamin C. Total ascorbic acid in the vegetable samples was analyzed using HPLC, after converting any dehydroascorbic acid (DHA) to ascorbic acid (AA) with H_2S. The method employed was that described for the analysis of Australian Aboriginal bushfoods (Brand Miller et al., 1993). The efficiency of conversion of DHA to AA by hydrogen sulphide treatment has been measured routinely as 90–95%. At all stages of preparation, samples were kept on ice. The final product was stored at −70°C for up to 30 days and samples were thawed immediately before analysis. Eluted peaks were identified by comparison with retention times of AA standards and confirmed with chromatographs of augmented samples. The mean recovery of vitamin C was 98.5% (range 91.3–108.5%). The coefficient of variation between samples was 6.4% and 2.0% within samples.

Table 4—Vitamin retention in steamed potato

Product description Sample	Mean end temp (°C)	Weight retention (%)	Percent vitamin retention Vitamin C mean (SEM)	5 MeTHF mean (SEM)
Cooked	87	100	100.0 (0.0)	100.0 (0.0)
Chilled	0	93.7	81.8 (7.9)	88.1 (7.6)
1 day chilled storage	2	92.7	72.4 (13.7)	87.7 (2.2)
3 day chilled storage	2	91.7	57.1 (14.6)	67.4 (11.3)
Room temp for 2 hr	15	89.8	70.7 (10.3)	77.8 (8.8)
Infra-red plated reheat	68	85.5	60.3 (5.8)	76.3 (12.8)
Infra-red bulk reheat	69	89.4	64.0 (10.9)	77.4 (3.9)
Conduction reheat	77	83.5	62.4* (18.2)	81.5* (9.8)
Warm-hold 30 min	75	99.4	64.6 (15.8)	82.6 (9.1)
Warm-hold 2 hr	67	99.4	61.0* (15.3)	77.4* (16.2)
Initial vitamin content/100g:			32.7 (1.9) mg	9.4 (0.4) µg

* One missing sample; mean calculated on n = 2

Table 5—Vitamin retention in mashed potato

Product description Sample	Mean end temp (°C)	Weight retention (%)	Percent vitamin retention Vitamin C mean (SEM)	5 MeTHF mean (SEM)
Cooked	61	100	100.0 (0.0)	100.0 (0.0)
Chilled	1	94.6	86.4 (3.9)	95.1 (2.6)
1 day chilled storage	3	93.6	73.9 (2.6)	91.4 (5.8)
3 day chilled storage	2	92.6	60.4 (6.3)	75.6 (12.3)
Room temp for 2 hr	14	90.7	65.4 (7.3)	76.8 (5.2)
Infra-red plated reheat	88	86.4	65.5 (7.5)	78.6 (5.5)
Infra-red bulk reheat	71	91.5	63.0 (2.8)	79.6 (13.5)
Conduction reheat	69	84.7	58.1 (7.9)	63.6 (6.5)
Warm-hold 30 min	72	99.2	61.0 (8.9)	85.2 (8.8)
Warm-hold 2 hr	76	96.1	44.1 (7.2)	67.5 (7.8)
Initial vitamin content/100g:			23.0 (1.0) mg	3.1 (0.6) µg

Folate. 5MeTHF was analyzed with the HPLC method used for the British total diet study (Farrar et al., 1992), modified by use of two C-18 columns in series, instead of a single column. Samples were stable at room temperature for at least 20 hr, but were stored at −18°C if not analyzed on the same day as deconjugation. The levels of 5MeTHF detected in samples treated with conjugase were on average 37% higher than in those without prior enzyme treatment. Eluted peaks were identified by comparison with retention times of 5MeTHF standards and confirmed with chromatographs of augmented samples. The mean recovery of 5MeTHF was 95.5% (range 90.0–101.2%). The coefficient of variation between samples was 8.2% and 3.7% within samples.

Calculation of nutrient retention

Duplicate assays of each sample were performed and the mean value used to calculate nutrient content. Percent retention of nutrients in each experimental product was adjusted to account for weight loss during processing, as recommended (Murphy et al., 1975), by the following formula:

$$\% \text{ Retention of nutrient} = \frac{\text{Nutrient content/g processed food} \times \text{g Food after processing}}{\text{Nutrient content/g cooked food} \times \text{g Food after cooking}}$$

'Processed' vegetables were defined as those samples taken after chilling, chilled storage, reheating or warm-holding. At each stage during processing, the weight of the vegetables was recorded. Weight loss was used to calculate percentage retention according to the formula, taking the freshly cooked product as the reference point of 100g.

Statistical analysis

The significance of differences in mean vitamin retention with the three reheating systems was analyzed using a random-block design analysis of variance, with three treatment groups (IR plated, IR bulk, or

Table 6—Vitamin retention in peas

Product description			Percent vitamin retention	
Sample	Mean end temp (°C)	Weight retention (%)	Vitamin C mean (SEM)	5 MeTHF mean (SEM)
Cooked	87	100	100.0 (0.0)	100.0 (0.0)
Chilled	2	95.5	71.3 (4.8)	90.6 (1.9)
1 day chilled storage	3	94.5	84.6 (2.1)	90.5 (1.6)
3 day chilled storage	2	94.5	67.9 (7.3)	85.2 (2.0)
Room temp for 2 hr	15	91.2	67.2 (6.8)	81.7 (7.3)
Infra-red plated reheat	71	88.5	52.8 (8.9)	90.8 (0.5)
Infra-red bulk reheat	71	93.2	36.6 (2.5)	81.0 (3.5)
Conduction reheat	74	85.3	37.5 (9.9)	88.6 (0.8)
Warm-hold 30 min	74	99.5	84.1 (4.9)	95.2 (1.3)
Warm-hold 2 hr	71	93.7	38.3 (18.3)	83.7 (1.7)
Initial vitamin content/100g:			13.2 (1.4) mg	14.3 (0.8) µg

Table 7—Vitamin retention in broccoli

Product description			Percent vitamin retention	
Sample	Mean end temp (°C)	Weight retention (%)	Vitamin C mean (SEM)	5 MeTHF mean (SEM)
Cooked	85	100	100.0 (0.0)	100.0 (0.0)
Chilled	1	94.8	67.5 (13.1)	83.9 (1.5)
1 day chilled storage	2	94.8	58.8 (6.9)	80.6 (2.0)
3 day chilled storage	2	92.2	56.4 (6.0)	82.4 (1.1)
Room temp for 2 hr	15	90.5	71.9 (17.9)	97.7 (2.0)
Infra-red plated reheat	73	90.4	69.0 (2.9)	84.9 (7.1)
Infra-red bulk reheat	84	92.7	37.6* (20.0)	76.4 (4.4)
Conduction reheat	71	90.8	51.8 (11.5)	93.6 (3.6)
Warm-hold 30 min	71	99.3	70.4 (11.8)	87.7 (5.0)
Warm-hold 2 hr	76	95.1	36.6 (9.9)	71.3 (2.5)
Initial vitamin content/100g:			30.0 (4.6) mg	22.1 (1.0) µg

* One missing sample; mean calculated on n = 2

Table 8—Vitamin retention in silverbeet

Product description			Percent vitamin retention	
Sample	Mean end temp (°C)	Weight retention (%)	Vitamin C mean (SEM)	5 MeTHF mean (SEM)
Cooked	89	100	100.0 (0.0)	100.0 (0.0)
Chilled	0	94.4	46.4 (13.3)	90.5 (3.6)
1 day chilled storage	1	94.4	35.1 (12.2)	83.4 (2.8)
3 day chilled storage	1	94.0	23.6 (10.8)	84.9 (3.1)
Room temp for 2 hr	15	92.9	28.2 (19.7)	80.4 (4.9)
Infra-red plated reheat	88	85.5	29.3 (17.4)	67.0 (4.0)
Infra-red bulk reheat	76	92.9	28.3 (14.4)	53.0 (6.5)
Conduction reheat	76	80.8	32.1 (20.8)	58.7 (1.4)
Warm-hold 30 min	71	98.8	45.0 (8.8)	81.9 (5.5)
Warm-hold 2 hr	68	98.4	31.7 (10.8)	43.3 (7.0)
Initial vitamin content/100g:			1.1 (0.1) mg	10.3 (1.5) µg

Table 9—Vitamin retention in pumpkin

Product description			Percent vitamin retention	
Sample	Mean end temp (°C)	Weight retention (%)	Vitamin C mean (SEM)	5 MeTHF mean (SEM)
Cooked	80	100	100.0 (0.0)	100.0 (0.0)
Chilled	1	94.1	72.0 (4.1)	58.6 (7.0)
1 day chilled storage	2	93.6	68.5 (0.6)	65.1 (3.7)
3 day chilled storage	2	93.6	41.1 (18.8)	32.3 (1.5)
Room temp for 2 hr	17	90.6	66.5 (12.5)	57.2 (7.6)
Infra-red plated reheat	76	88.1	50.8 (14.6)	71.5 (14.9)
Infra-red bulk reheat	81	92.0	71.4 (12.5)	48.0 (3.6)
Conduction reheat	76	84.7	52.0 (7.1)	65.2 (10.8)
Warm-hold 30 min	70	99.4	71.2 (6.5)	64.6 (10.9)
Warm-hold 2 hr	73	97.4	47.6 (11.4)	64.2 (14.0)
Initial volume content/100g:			9.6 (0.2) mg	9.0 (0.3) µg

conduction reheating) and seven replicate blocks (the vegetables). Comparisons between retention of vitamins in reheated chilled food and food that had been held hot for 30 min or 2 hr were analyzed using two-tailed paired *t* tests. The significance of losses after holding at room temperature for 2 hr (sample 5) or at 3°C for 2 additional days (sample 4) compared to food stored chilled for 24 hr (sample 3) was determined by one-tail paired *t* tests. The correlation between rates of vitamin loss and initial vitamin concentration in the vegetables was tested by calculating Spearman's rank correlation coefficient. Statistical analyses were performed using the Statview Student statistical program (Abacus Concepts, 1991).

RESULTS & DISCUSSION

MEAN TEMPERATURES of the vegetables were measured (Table 2) at each of the sampling stages. The final temperatures after reheating (samples 6–8) were not different when analysed by one-factor ANOVA ($p = 0.327$). Vitamin retention results from all seven vegetables were combined (Table 3). Retention of the two vitamins was also reported separately for each vegetable (Table 4–10).

Losses during chilling and storage

During rapid chilling of the cooked vegetables, a substantial loss occurred for vitamin C (mean reduction 28%) but a smaller decline in the 5MeTHF content (17%). Chilled storage below 3°C for 3 days caused a further linear loss of vitamin C (7.9%/day $r = 0.995$). 5MeTHF levels also declined, but not as rapidly (4.8%/day, $r = 0.937$) over the 3 days of chilled storage. Table 11 shows the rates of loss during chilled storage for each of the vegetables in the study.

Effect of plating at room temperature

Possible temperature abuse during plating was simulated by leaving plated food at room temperature (~ 20°C) for 2 hrs, with a resulting average rise in food temperatures to 15°C. This prolonged holding above 10°C led to additional losses of 4% of vitamin C and 3% of 5MeTHF. Neither of these changes was significant ($p = 0.157$ for vitamin C; $p = 0.133$ for 5MeTHF).

Losses during reheating

Reheating of vegetables after 1 day of chilled storage resulted in a mean loss of 16% of vitamin C and 8% of 5MeTHF. ANOVA indicated that the percent retention of both vitamins was comparable for all 3 reheating methods ($p > 0.4$). However the extent of vitamin loss varied between vegetables. Losses of vitamin C were greatest with peas (42%) and carrots (27%) and least with boiled potatoes (6%) and pumpkin (9%). Losses of 5MeTHF were greatest with silverbeet (24%) and mashed potato (17%) and least with peas and broccoli (4% each).

Losses during warm-holding

Considerable losses of both vitamins occurred during warm-holding at 72°C. Vitamin C levels fell by 26%/hour ($r = 0.856$) and 5MeTHF declined by 14%/hr ($r = 0.863$). Median values for losses of vitamin C during warm-holding (36% at 30 min and 60% after 2 hr) were similar to reports by others (Briant et al., 1946; Ang, et al., 1975; Hill et al, 1977; Faerden, 1983; Carlson and Tabacchi, 1988). The rates of vitamin losses in each vegetable during warm-holding were compared (Table 11).

Comparisons between cook/chill and cook/hot-hold systems

Under normal handling conditions, vitamin losses in vegetables were greater with cook/chill than cook/hot-hold foodservice systems. Comparison of the vitamin C content showed less retained in the cook/chill system compared to food held hot for 30 min (50% vs 65%). More was retained compared to food held hot for 2 hr ($p < 0.05$). Degradation of vitamin C in food during warm-holding at 70 to 130°C followed first order kinetics (Paulus, 1979). Therefore, assuming linear losses, the time at which losses in the two systems were similar would be after 91 min of warm-holding.

Table 10—Vitamin retention in carrots

Product description			Percent vitamin retention	
Sample	Mean end temp (°C)	Weight retention (%)	Vitamin C mean (SEM)	5 MeTHF mean (SEM)
Cooked	92	100	100.0 (0.0)	100.0 (0.0)
Chilled	1	95.3	82.1 (8.1)	69.9 (13.2)
1 day chilled storage	2	92.0	69.4 (28.4)	69.7 (4.2)
3 day chilled storage	2	89.9	37.8 (5.3)	51.2 (4.8)
Room temp for 2 hr	17	89.4	66.2 (23.2)	76.7 (0.2)
Infra-red plated reheat	72	80.9	52.7 (2.8)	73.9 (15.9)
Infra-red bulk reheat	76	89.1	34.9 (0.8)	71.5 (3.9)
Conduction reheat	75	83.1	33.8 (2.5)	75.4 (11.9)
Warm-hold 30 min	72	99.1	50.1 (9.0)	65.7 (1.0)
Warm-hold 2 hr	73	95.8	24.3 (6.0)	71.1 (6.6)
Initial vitamin content/100g:			1.2 (0.2) mg	6.7 (0.6) µg

Results for 5MeTHF were similar to those for vitamin C. Retention in reheated chilled food was less than in food held hot 30 min (74% vs 81%) and not different from that in food held hot 2 hr (74% vs 68%). Assuming linear losses during warm-holding, the break-even point between the systems was again ~ 1½ hr. Williams and Brand Miller (1993) reported that the average warm-holding time in NSW hospitals with cook/hot-hold foodservices was 45 min. Therefore it is unlikely that retention of vitamin C or 5MeTHF in vegetables served to patients would increase for most hospitals changing to a cook/chill system.

However, in this simulation experiment, losses in the cook/chill system were minimized by holding the food chilled for only 1 day and sampling the food immediately after reheating. In many hospitals some food would be held chilled for 3–5 days before reheating. Furthermore, if food is reheated in bulk in a central kitchen before service and distribution, there would be additional losses caused by further warm-holding before delivery to the consumer. The losses of 7.9% of vitamin C and 4.8% of 5MeTHF/day measured over 3 days could be assumed to continue up to 5 days. Then increasing the chilled storage time from 1 to 5 days would reduce vitamin levels in vegetables by a further 30% (vitamin C) and 19% (5MeTHF) before reheating. This would result in overall retentions of 28% and 58%, respectively, in the reheated product—substantially less than retention measured in vegetables held hot as long as 2 hr.

These findings confirmed those of Mikkelsen (1985), who reported that the ascorbic acid content of potatoes held hot for 90 min was equivalent to that in food reheated after one day chilled storage, but higher than in food reheated after 3 days of chilled storage. They also agreed with those of Ang et al., (1975), who reported the retention of thiamin in reheated chilled food was comparable with fresh product held hot for 90 min.

One reason for the lower retention in the cook/chill system was the sometimes unrecognized but notable vitamin destruction that could occur during the chilling process. Losses of vitamin C during initial chilling of cooked food (28%) were similar to those reported by Kossovitsas et al. (1973) who found losses of 31% of the ascorbic acid during chilling of cooked broccoli. The only reported study of retention of total folate during chilling indicated a loss of 18.5% in baked potato (Augustin et al., 1980), comparable to the mean loss of 17% in the seven vegetables we found.

Two factors may contribute to such losses: the period of time that the food remained at elevated temperatures during cooling, and the rapid air movement in the blast chiller that might increase oxidative processes. Even with efficient chillers, the vegetables usually remained above 10°C for at least 45 min during cooling. Thus some oxidative changes, like those occurring during warm-holding, would occur in the early stages of chilling when the food was still relatively hot.

Effects of different reheating systems

A second conclusion was that the three tested reheating systems (infra-red bulk, infra-red plated and conduction plated) were comparable in their effects on nutrient retention. Despite varying heating times and temperatures achieved with the three systems, no significant differences occurred among percent retention of vitamin C or 5MeTHF when the vegetables were reheated in different ways, either as plated meals or in bulk. This confirmed the findings of other studies that compared reheating methods. Unklesbay et al. (1983) reported no difference in vitamin C retention in potatoes and tomatoes reheated by IR or convective heating. Bognar et al. (1990) studied convection, microwave, IR and water-bath bulk reheating of a number of vegetables and concluded that 'the thermal stress in the different reheating systems was nearly the same despite different heating times'. The only other study that examined effects of another conduction reheating method (the 3M system) measured AA and thiamin retention in potatoes and peas and found no difference with conduction, convection or microwave reheating methods (Dahl-Sawyer et al., 1982).

One study of reheating on folate retention in vegetables used a microwave oven to reheat 90g individual portions of potato and reported no change in total folate (Augustin et al., 1980). Microwave reheating is usually less destructive compared with other heating methods (Cross and Fung, 1982). Thus, the greater degree of folate loss we found (average = 8%) was likely due to use of IR and conduction ovens. They require longer heating times, and may expose some of the food to higher temperatures during reheating.

Table 11—Vitamin losses in cooked vegetables during chilled storage (%/day) and warm-holding (%/hr)

	Chilled storage (3°C)		Warm-holding (72°C)	
Vegetable	Vitamin C	5MeTHF	Vitamin C	5MeTHF
Carrots	14.9	6.7	33.1	10.3
Pumpkin	10.8	9.9	30.8	8.0
Mashed potato	8.4	6.7	29.6	13.6
Steamed potato	8.2	6.4	28.3	27.7
Silverbeet	7.3	1.5	24.1	15.2
Broccoli	3.3	0.3	23.8	13.8
Peas	2.2	1.9	15.5	9.5
Mean	7.9	4.8	26.4	14.0

Folate losses in foodservices

The losses of 5MeTHF found in the 7 vegetables (17% during chilling; 4.8%/day in chilled storage; 7.8% during reheating; 14.0%/hr of warm-holding) indicated that folate was second only to vitamin C in its sensitivity to destruction during foodservice handling. The losses were linear with time during warm-holding ($r = 0.863$) and chilled storage ($r = 0.937$). The implications of these findings for losses in total folate is uncertain. 5MeTHF is one of the least stable forms of the vitamin, with a half-life at 100°C of <10 min. 5-Formyltetrahydrofolate, the other main form found in vegetables, is stable for up to 10 hr (Paine-Wilson and Chen, 1979). Therefore most losses of folate in vegetables during processing are likely due to oxidation of 5MeTHF. If other folate forms are not as readily oxidized, the loss of total folate activity may only be half the rates reported here. Note that the levels of 5MeTHF in the vegetables were only around half the expected total folate content. However, losses found were similar to those measuring total folate by microbiological assay (Augustin et al., 1980, 1981; Bender, 1985).

Differences among vegetables

While rates of loss of vitamin C and 5MeTHF during both warm-holding and chilled storage were linear with time, they varied among the vegetables (Table 11). Rates were not correlated with initial concentrations of the vitamins, but appeared more influenced by other characteristics, such as physical form of the food. For example, those with the greatest losses had the largest cut surface areas.

The ranking of susceptibility to losses during chilled storage moderately correlated between the two vitamins (r_s = 0.83; p = 0.042): carrots, pumpkin and mashed potato had the highest losses; peas and broccoli the lowest. The pattern of loss was different during warm-holding and there was no correlation between the two vitamins. Rates of vitamin C losses were similar in all vegetables. The losses of 5MeTHF varied more; they were low in steamed potato (9.5%/hr) and greatest in silverbeet (27.7%/hr).

Comparisons with published vitamin analyses

The analyzed vitamin C values confirmed the published values in Australian food tables (Commonwealth Department of Community Service and Health, 1989), with exception of silverbeet, which was unexpectedly low. Because silverbeet was purchased finely chopped probably considerable oxidative losses had occurred in the fresh product before processing. Chopped spinach can lose up to 25% of initial vitamin C after 24 hr storage at 12°C (Fennema, 1977). There could also have been considerable losses by leaching into cooking water.

No published values were found for the 5MeTHF content of the foods we studied except broccoli. Using reverse-phase liquid chromatography, Gregory (1984) reported that the 5MeTHF content of broccoli was 20 µg/100 g and our results (22 µg/100g) confirmed that. In an interlaboratory comparison of determination of folates, the mean (±SD) reported values from 2 unidentified laboratories using HPLC to determine 5MeTHF in standardized freeze-dried Brussels sprouts after chicken pancreas deconjugation were 390 ± 54 and 981 ± 100 µg/100 g (Finglas et al., 1993). Using the same standardized material obtained from the AFRC Institute of Food Research, the mean value we measured calculated from 6 replicate extractions, was 554 ± 46 µg/100g. Thus, the method appeared to give results in the same range of the limited published data.

Compared to British figures for total folate of vegetables, analyzed by microbiological assay (Holland et al., 1991), the proportion contributed by 5MeTHF, (as we measured) averaged 49% (peas 31%, broccoli 34%, carrots 44%, potatoes 47%, and pumpkin 90%). In a separate study during method testing, frozen chopped Belgian spinach and freeze-dried Brussels sprouts were analyzed for 5MeTHF; the values were 38% and 56% of published total folate values, respectively. These proportions were similar to reported values of 35% in broccoli (Gregory, 1984) and 47% in Brussels sprouts (Finglas et al. 1993).

CONCLUSION

USING THE TWO most sensitive indicators of nutrient loss, consistent results indicated that the nutritional quality of vegetables would be better preserved with a conventional cook/hot-hold foodservice than a cook/chill system. This conclusion assumes that warm-holding times would be restricted to < 90 min, after which time the cook/chill system may give better retention. In most Australian hospitals, where normal warm-holding times are around 45 min, the cook/hot-hold system would be nutritionally preferred.

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Accumulation of Iron in Lactic Acid Bacteria and Bifidobacteria

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ABSTRACT

Lactobacillus acidophilus, L. delbrueckii var. *bulgaricus, L. plantarum* and *Streptococcus thermophilum*, all used extensively in the food industry, were tested for their ability to internalize and/or oxidize ferrous iron (Fe^{2+}). For comparison some experiments were performed with bifidobacteria, *B. thermophilum* and *B. breve*. All organisms except *L. bulgaricus* could transport Fe^{2+} into the cell, where it was partially oxidized to the ferric form (Fe(III)). In addition, *L. acidophilus* and *L. bulgaricus* could oxidize Fe^{2+} to Fe(III) extracellularly through the elaboration of H_2O_2 into the medium when the experiments were carried out in air. *L. bulgaricus* elaborated H_2O_2 only in the presence of glucose, whereas *L. acidophilus* released H_2O_2 in absence of glucose. We concluded that lactic acid bacteria, like bifidobacteria, can exert some beneficial effects in animal organisms, or in food processing and storage, by making Fe^{2+} unavailable to harmful microorganisms.

Key Words: ferrous iron; lactic acid bacteria; bifidobacteria

INTRODUCTION

IT HAS BEEN HYPOTHESIZED that ferrous iron accumulation by bacteria enables them to retrieve iron from the medium for growth under anaerobic conditions (Kammler et al., 1993). Another effect of ferrous iron assimilation by such intestinal microorganisms as bifidobacteria may be prevention of its use by other, especially pathogenic microorganisms (Bezkorovainy and Solberg, 1989). In such cases, the amount of iron taken up may be very high, especially because the internalized iron may be oxidized by intracellular ferroxidases and precipitated as $Fe(OH)_3$. This would serve to maintain Fe^{2+} concentration inside the cell at a low level thus enhancing its entry into the cell from the medium (Kot et al., 1994).

Bifidobacteria, as well as other lactic acid-producing fermentative microorganisms, have been used extensively as food additives, and various health benefits have been ascribed to them (Kurmann and Rasic, 1991; Davidson and Hoover, 1993). Certain health benefits may be due, in part, to the ability of such organisms to withhold iron from pathogens. This phenomenon, which is usually associated with the transferrin-type iron-binding proteins, has been termed nutritional immunity (Weinberg, 1986; Bezkorovainy, 1980).

Iron accumulation by fermentative lactic acid-producing bacteria has been studied thoroughly only in bifidobacteria. In view of the importance of other organisms such as lactobacilli in food industry and medicine, there is need to investigate their iron-accumulating activities as well. Consequently, the following organisms, typical of this group, were chosen for the present study: *Lactobacillus acidophilus, L. delbrueckii* var. *bulgaricus, L. plantarum* and *Streptococcus thermophilum*. Our objective was to determine their ferrous iron-handling properties and compare them to those of *Bifidobacterium breve* and *B. thermophilum*, whose Fe^{2+} uptake and oxidizing activities have been reported to some extent (Bezkorovainy and Solberg, 1989; Kot and Bezkorovainy, 1991, 1993b). Note that some lactobacilli are capable of elaborating hydrogen peroxide into the medium in the presence of O_2 (Condon, 1987), and this may affect the mode of iron accumulation by such bacteria as well.

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MATERIALS & METHODS

Microorganisms

All microorganisms were obtained from American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852). They were *L. acidophilus* (ATCC 4356), *L. delbrueckii* var. *bulgaricus* (ATCC 11842), *L. plantarum* (ATCC 14917), *Streptococcus thermophilum* (ATCC 19258), *B. breve* (ATCC 15700) and *B. thermophilum* (ATCC 25866). They were grown under anaerobic conditions in the Trypticase-Phytone-Yeast Extract (TPY) medium described by Scardovi (1986), except bifidobacteria, which were grown in the modified TPY medium described by Kot and Bezkorovainy (1991). Microorganisms were grown in volumes of 120 mL for 18 hr, at which time resting phases were achieved. Absorbances at that point at 610 nm were 1.2, except for *L. plantarum* and *S. thermophilum*, which showed absorbances of 0.80.

Bacterial cells were disrupted by sonication or the French pressure cell as previously described (Kot and Bezkorovainy, 1993b). The purpose of this procedure was to prepare particulate fractions of microorganisms for a determination of their ability to oxidize Fe^{2+} to Fe(III). The extent of cellular disruption was identical by both methods as evaluated by a decline in lactic acid production. This was previously shown to be the case with bifidobacteria (Kot and Bezkorovainy, 1993b).

Iron accumulation assays

Cells were harvested from growth media by centrifugation at 6000 × *g* for 10 min at 4°C (range 3–5°C), the spent medium was decanted and the cells washed once with ice-cold 0.9% NaCl. Cells were then suspended in ice-cold 0.1M 3,3-dimethylglutarate buffer at pH 6.5, which also contained 0.4g KCl, 8.0g NaCl and 0.14g $CaCl_2$/L. The A_{610} was then adjusted to 1.2 with this buffer. Depending on experimental design, the suspension buffer either contained freshly-added 2 mg/mL glucose or contained no carbon source.

Iron accumulation experiments were performed on bacterial cell suspensions as follows: Cell suspensions were warmed to 37°C (or they remained at 0°C on ice), and then 10 mM $FeSO_4$ labeled with $^{59}Fe^{2+}$ (stock solution) was added to final Fe^{2+} concentrations of 10 μM to 200 μM. The stock solution was made up in water and contained 1 mg/mL ascorbic acid as an antioxidant. Cells were incubated with shaking for up to 60 min in air or at low pO_2. At various time intervals, 5 mL samples were removed, cooled to 0°C in an ice-bath, and centrifuged at 4°C at 6000 × g for 10 min. The cells were then washed with 5 mL ice-cold 0.1M acetate buffer at pH 5.0 and counted. Occasionally, cells were washed with ice-cold 2 mM $FeSO_4$ (nonradioactive) in 0.1M acetate buffer at pH 5.0 following the plain acetate buffer wash. The washing continued for 30 min. with shaking; its purpose was to assess surface binding of $^{59}Fe^{2+}$ by bacteria.

Iron accumulation by bacterial cells was expressed as nmoles of radioactive iron that was associated with a cell pellet. A "pellet," which was counted, was defined as that amount of cells, present in 5 mL of a bacterial suspension with an A_{610} = 1.2. For bifidobacteria, this amounted to 2.5 mg dry weight (Bezkorovainy and Solberg, 1989). A "pellet equivalent" refers to the supernatant of a "pellet."

Iron accumulation by a pellet of cells was the sum total of iron that had been internalized by the cells, iron absorbed unto the cell surface, or ferrous iron that had been oxidized to Fe(III) in the medium and co-centrifuged out with the bacterial pellet. Since the stock $FeSO_4$ solutions contained ascorbate, the amount of Fe^{2+} oxidized non-specifically to Fe(III) was not measureable (Bezkorovainy et al., 1988).

Occasionally, it was necessary to test cellular supernatants for the presence of H_2O_2 and its Fe^{2+}-oxidizing activity. Since H_2O_2 would also

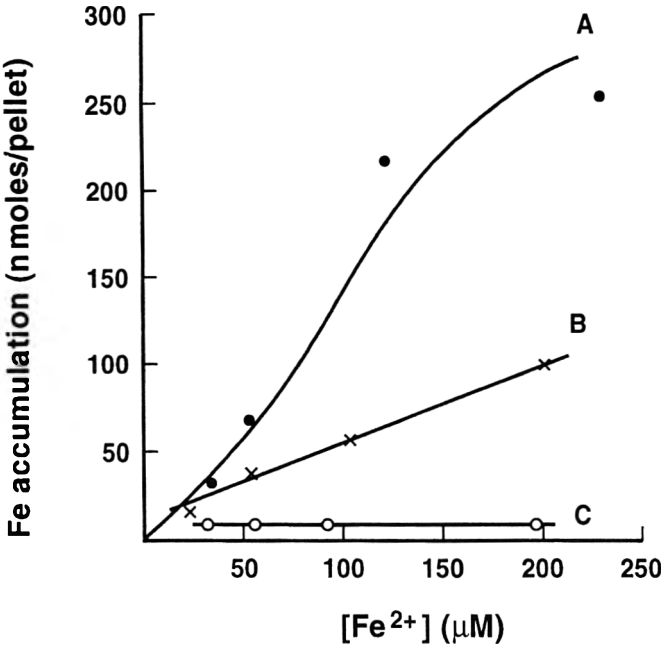

Fig. 1—Relation of iron accumulation by *L. plantarum* to the concentration of Fe^{2+}. Incubation with $^{59}Fe^{2+}$ 1 hr at pH 6.5 in air. (A) incubation in presence of 2 mg/mL glucose at 37°C; (B) in absence of glucose at 37°C; (C) in presence of glucose at 0°C.

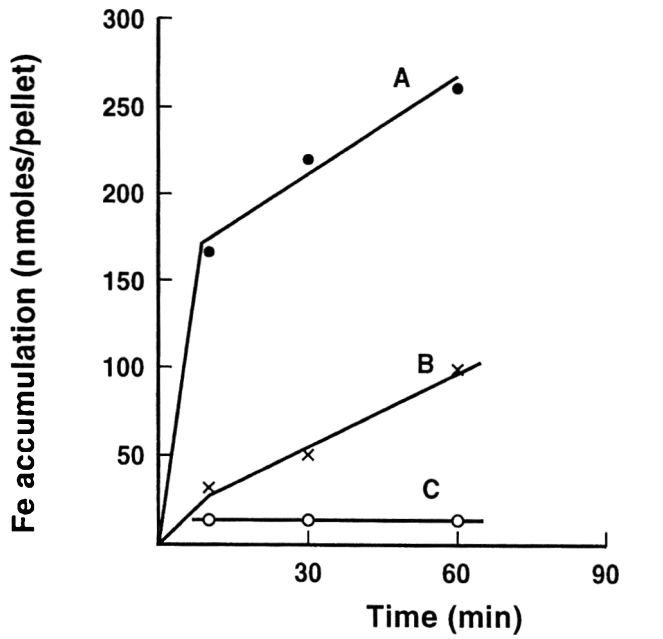

Fig. 2—Time-course studies on iron accumulation by *L. plantarum*. Incubations in air at pH 6.5. (A) incubation in presence of 2 mg/mL glucose at $[Fe^{2+}]$ = 230 μM and 37°C; (B) in absence of glucose at $[Fe^{2+}]$ = 201 μM and 37°C; (C) in presence of glucose at $[Fe^{2+}]$ = 199 μM and 0°C.

react with ascorbate, this agent was omitted from the Fe^{2+} solutions used. In such cases, the assay was run for 10 min., since the reaction of H_2O_2 with Fe^{2+} was almost instantaneous. The amount of Fe^{2+} oxidized was then determined by the ferrozine reaction, which gave a purple color with Fe^{2+} only. The amount of Fe^{2+} oxidized was expressed in terms of nmoles/5mL of cell supernatant (pellet equivalent). Nonspecific oxidation of ferrous iron at pH 6.5 in the absence of ascorbate amounted to 10–15% when Fe^{2+} concentration in the medium was 200 uM, and it varied from one experiment to another. All data on Fe^{2+} oxidation by cell supernatants were corrected for this non-specific oxidation by appropriate controls.

Iron accumulation studies at low pO_2 (53 mm Hg) were done using N_2 as previously described (Kot et al., 1994).

Source of chemicals

Most chemicals and various analytical kits were obtained from Sigma Corporation (P. O. Box 14508, St. Louis, MO 63178). $^{59}Fe^{2+}$ was purchased from DuPont Laboratories (549 Albany St., Boston, MA 02118). Trypticase and phytone, used in the preparation of the TPY medium, were purchased from BBL Microbiology Systems (Cockeysville, MD 21030).

Analytical procedures

^{59}Fe was counted in a Beckman Instruments Gamma 4000 counter (2500 Harbor Blvd., Fullerton, CA 92634). Colorimetric assays of ferrous iron via the ferrozine reaction were a modification of a procedure designed for serum iron determination by the Sigma Corporation, using kit No. 565: 0.5 mL of the sample was mixed with 2 mL of 0.1M acetate buffer at pH 5.0, then 0.05 mL of 0.85% ferrozine in 0.1M acetate buffer at pH 5.0 was added. After incubating the reaction mixture for 30 min at 37°C, it was read at 560 nm using authentic $FeSO_4$ as a standard. Hydrogen peroxide was determined using modifications of Sigma Corporation procedures for serum cholesterol and/or glucose determinations (kits No. 352 and 510, respectively). Both procedures result in production of H_2O_2 from these analytes when used in the clinical biochemistry laboratory. The H_2O_2 was then quantitated via a peroxidase-dependent oxidation of the O-dianisidine dye in case of glucose and quinoneimine dye in case of cholesterol assay. In our case, instead of using serum, bacterial supernatants were used. Kit 510 could not be used if the medium contained glucose.

L(+)-lactate was determined using Sigma Corporation's kit No. 826-UV; it determined that lactate isomer only.

RESULTS

Accumulation of iron by lactic acid bacteria and bifidobacteria

All lactic acid cultures and bifidobacteria, when incubated with $^{59}Fe^{2+}$, showed accumulation of iron. Such accumulations were $[Fe^{2+}]$ and time dependent, as shown, for example, for *L. plantarum* (Figs. 1, 2). The results for one Fe^{2+} concentration following a 60 min. incubation at 37°C were compared (Table 1). These showed that in the absence of glucose, iron accumulations were low. An exception was the case with *L. acidophilus* and *L. bulgaricus*, which accumulated high amounts of iron under those conditions (168% and 68.6% of that in the presence of glucose, respectively). A notable amount of Fe^{2+} could be eluted from these organisms by cold 2 mM $FeSO_4$ at 0°C, which may indicate surface binding.

Elaboration of hydrogen peroxide into the medium by lactic acid bacteria

Possible production of hydrogen peroxide by bacteria was tested as follows: Cells were incubated in the presence or absence of glucose at 37°C for 30 min. in air. They were then centrifuged out at 4°C, and their supernatants were tested for the presence of hydrogen peroxide and ability to oxidize Fe^{2+} to Fe(III). Iron concentrations were 10 uM to 200 uM, and the reaction was allowed to take place for 10 min at 37°C (Table 2). Results showed that H_2O_2 was not produced by bifidobacteria and *L. plantarum*, and that only minor amounts thereof were produced by *S. thermophilum*. In *L. bulgaricus*, hydrogen peroxide was produced in the presence of glucose, but little in its absence. The reverse was true for *L. acidophilus*. Those supernatants, which contained H_2O_2 also oxidized Fe^{2+} to Fe(III).

Iron accumulation by lactic acid bacteria and bifidobacteria at 0°C and after heating

Iron accumulation by bacteria at 0°C was almost instantaneous (Table 3). In *L. plantarum* and *S. thermophilum*, most iron

Table 1—Iron accumulation by various lactic acid bacteria and bifidobacteria (in nmoles/pellet) as affected by glucose (2 mg/mL), and % of iron washed off by 2 mM $FeSO_4$[a]

Organism	Iron accumulation in presence of glucose[b]			Iron accumulation in absence of glucose[c]			Fe without: Fe with glucose
	$[Fe^{2+}]$ (μM)	Fe accumulated	Washed off	$[Fe^{2+}]$ (μM)	Fe accumulated	Washed off	
L. acidophilus	185 ± 6.54	231 ± 64.6	14.1 ± 1.33	196 ± 9.67	388 ± 95.8	23.4 ± 1.92	1.73 ± 0.445
L. bulgaricus	199 ± 4.61	354 ± 70.7	20.4 ± 5.42	207 ± 8.25	243 ± 102	21.8 ± 9.86	0.666 ± 0.214
L. plantarum	206 ± 19.0	323 ± 121	8.45 ± 6.92	202 ± 14.0	64.3 ± 36.1	29.6 ± 6.18	0.155 ± 0.0697
S. thermophilum	192 ± 4.24	171 ± 34.4	13.0 ± 5.66	200 ± 6.42	102 ± 42.1	16.6 ± 12.3	0.557 ± 0.137
B. thermophilum	201 ± 8.33	274 ± 77.8	9.63 ± 3.25	199 ± 8.19	59.8 ± 50.8	—[d]	0.172 ± 0.104
B. breve[e]	197	239	19.0	194	18.6	18.9	0.0778

[a] Incubations at 37°C for 60 min in air.

[b] Lactate production was as follows (in μg/mL): 773 ± 90.3 for *L. acidophilus*; 235 ± 82.8 for *L. plantarum*, 1043 ± 90.5 for *S. thermophilum*; 236 ± 37.8 for *B. thermophilum*; and 267 for *B. breve*. No data for *L. bulgaricus*, since it produces no measurable L(+)-lactate.

[c] Lactic acid production was nil for all organisms.

[d] Not done.

[e] Single experiment.

Table 2—Lactic acid bacteria and bifidobacteria production of hydrogen peroxide (in μM) and capacity of supernatants to oxidize Fe^{2+} to Fe(III) (in % of initial $[Fe^{2+}]$)

Microorganism	Hydrogen peroxide produced[a]		Fe^{2+} oxidized by supernatant[b]	
	With glucose[c]	No glucose	With glucose	No glucose
L. bulgaricus	833	90.0	79.5	27.0
L. acidophilus	84.0	360	42.6	83.3
L. plantarum	0.00	0.00	1.90	0.00
S. thermophilum	62.0	45.0	14.0	8.00
B. breve	0.00	0.00	12.0	10.0
B. thermophilum	0.00	0.00	8.00	10.0

[a] Incubation at pH 6.5 in air at 37°C for 30 min. H_2O_2 measured in pellet supernatants (pellet equivalents).

[b] Iron concentrations were 219 ± 20.6 μM in absence of antioxidant. Assay carried out for 10 min. All values corrected for nonspecific oxidation of Fe^{2+} in the pH 6.5 buffer.

[c] Glucose concentration 2 mg/mL.

Table 3—Iron accumulation by lactic acid bacteria as affected by 2 mg/mL glucose at 0° C, and % washed off by 2 mM $FeSO_4$ (nmoles/pellet)[a]

Organism	$[Fe^{2+}]$ (μM)	Iron accumulation with glucose		Iron accumulation without glucose	
		Accumulated	Washed off	Accumulated	Washed off
L. acidophilus	100	31.0	29	73.6	32
L. bulgaricus	112	80.4	30	8.40	55
L. plantarum	100	7.65	100	2.18	100
S. thermophilum	150	7.64	40	10.6	74

[a] Incubations were carried out for 1 hr in air. Lactate production was nil in all cases.

accumulated could be removed by washing with 2 mM $FeSO_4$, indicating that it was surface bound. The same was true of *L. bulgaricus* in the absence of glucose. However, *L. acidophilus* in the absence of glucose and *L. bulgaricus* in its presence, could accumulate relatively large amounts of iron at 0°C, though less than at 37°C.

Iron accumulation was also measured in heated cells. The heating was at 80°C for 15 min either in the presence or absence of glucose. After cooling, $^{59}Fe^{2+}$ was added to a final concentration of 200 μM, and the cells were incubated further for 60 min at 37°C in air. Iron accumulation was determined and results compared (Table 4). No lactate was produced by heated cells, nor in the unheated controls in the absence of glucose. The non-H_2O_2 producers, *L. plantarum, S. thermophilum* and *B. thermophilum*, showed greatly diminished abilities to accumulate iron after heating. Of that accumulated, most was eluted by 2 mM $FeSO_4$. Iron accumulation by heated *L. acidophilus* and *L. bulgaricus* was lower than that in unheated cells, though substantial accumulations were still observed.

Effect of low pO_2 on accumulation of iron by lactic acid bacteria

Iron accumulation experiments with lactic acid bacteria were performed at decreased pO_2 as described (Kot et al., 1994) (Table 5). Data indicated that for all four bacterial species, iron accumulation was lowered when pO_2 was low. Hydrogen peroxide was not measured in supernatants of *L. plantarum* and *S. thermophilum*, since they did not produce it in air. H_2O_2 is known to be produced only in an O_2 atmosphere (Condon, 1987). This was confirmed for *L. bulgaricus*, where H_2O_2 production at low pO_2 was only 5% that observed in air.

Effect of magnesium

In a previous investigation on bifidobacteria, it was reported that Mg^{2+} could inhibit iron accumulation at ≥200 uM and higher (Kot and Bezkorovainy, 1993a). The same type experiments were performed with all four lactic acid bacterial species, and no effect by Mg^{2+} was found (data not shown). Lactate production was unaffected by Mg^{2+}.

Oxidation of Fe^{2+} by particulate fractions of lactic acid bacteria

Particulate fractions of bifidobacteria (prepared either by sonication or by the French pressure cell) showed Fe^{2+}-oxidizing activities and were assumed to contain putative ferroxidases (Kot et al., 1994). Particulate fractions of lactic acid bacteria were prepared and tested for abilities to oxidize and precipitate Fe^{2+} (Table 6). Considerable amounts of iron were oxidized by all organism particulate fractions, except for *L. bulgaricus*, and only 6.9% to 29% of the precipitated iron could be washed off by 2 mM $FeSO_4$. Lactic acid production by such fractions was low compared to that of intact organisms (see Table 1), indicating nearly complete cellular disruption.

DISCUSSION

BIFIDOBACTERIA have been shown to remove ferrous iron from media by internalizing it and then, when oxygen was present, partially oxidizing it to the insoluble $Fe(OH)_3$ by putative intracellular ferroxidases (Kot et al., 1994). The same phenomenon was apparent in *L. plantarum, L. acidophilus* and *S. thermophilum*. This hypothesis is supported by the fact that iron accumulation in those organisms was sharply lowered at low pO_2, at 0°C and in heated cells. The putative ferroxidases were most likely associated with the cellular particulate fractions (Table 6). In *L. acidophilus*, this mechanism would be, of course, superimposed upon that involving hydrogen peroxide effects. Hydrogen peroxide was apparently elaborated into medium, albeit in lower amounts, even at 0°C and by heated cells resulting in Fe^{2+} oxidation (Tables 3 and 4). An exception to this may be *L. bulgaricus*, which showed minimal amounts of ferroxidase activity in its particulate fraction. Its ferrous iron-deactivating activity may thus be associated only with H_2O_2 production.

The removal of Fe^{2+} from the medium by lactic acid bacteria was not affected by Mg^{2+}, whereas in bifidobacteria, there was a strong inhibition of Fe^{2+} uptake by Mg^{2+} (Kot and Bezkorovainy, 1993a). This difference may indicate a difference in Fe^{2+} permease function among those types of bacteria.

Table 4—Iron accumulation by heated lactic acid bacteria and *B. thermophilum* (in nmoles/pellet) and lactic acid production by heated cells (% of unheated controls)

	Iron uptake						
	with glucose				without glucose[a]		
Organism	Heated cells	Washed off by 2 mM $FeSO_4$ (%)	Unheated controls	Lactate produced	Heated cells	Washed off by 2 mM $FeSO_4$ (%)	Unheated controls
L. acidophilus	253	19.0	196	10.2	260	18.5	467
L. bulgaricus	159	37.9	323	—[b]	142	38.4	125
L. plantarum	21.3	36.4	468	14.9	29.2	34.5	25.2
S. thermophilum	58.0	36.4	186	14.3	58.0	36.2	114
B. thermophilum	19.5	56.7	181	11.5	20.8	38.7	13.4

[a] Lactate production was nil in both heated cells and unheated controls.
[b] Experiment not done[b]

Table 5—Iron accumulation in lactic acid bacteria and *B. thermophilum* at low pO_2 as affected by 2 mg/mL glucose (in % of that seen in air)[a]

Microorganism	With glucose	Without glucose
L. bulgaricus[b]	3.6	6.3
L. acidophilus[c]	6.2	2.5
L. plantarum	27	16
S. thermophilum	17	16
B. thermophilum	29	46

[a] All incubations with 200 μM Fe^{2+} for 60 min at 37°C.
[b] Produces H_2O_2 in air and in presence of glucose.
[c] Produces H_2O_2 in air and in absence of glucose.

Table 6—Deposition of iron by particulate fractions of lactic acid bacteria (nmoles/mg protein), percent washed off by 2 mM $FeSO_4$ and lactate produced (in μg/mL)[a]

Organism	Iron deposited	Washed off	Lactate produced
L. acidophilus	109	29	48.0
L. bulgaricus	7.69	60	—[b]
L. plantarum	25.4	20	45.0
S. thermophilum	58.1	7.0	276

[a] Particulate fractions from one bacterial pellet incubated with 100 μM $^{59}Fe^{2+}$ for 60 min at 37°C in air.
[b] Experiment not done; *L. bulgaricus* products D(−) lactic acid that cannot be measured by this method.

Lactic acid bacteria could apparently remove Fe^{2+} from its environment, either through H_2O_2 production or internalization-oxidation. We could thus conclude that these organisms, like bifidobacteria, may participate in the nutritional immunity phenomenon (Weinberg, 1986). Such Fe^{2+} removal may take place in the gastrointestinal tract through the consumption of fermented or supplemented milk products, or this may occur in food storage or processing situations. Beneficial effects observed when lactic acid bacteria or bifidobacteria are used in foods may be due, in part, to their effectiveness in withholding iron from pathogens and/or putrefactive organisms.

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Cloning and Expression of a Limonene Degradation Pathway from *Bacillus stearothermophilus* in *Escherichia coli*

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ABSTRACT

The pathway encoding limonene catabolism was cloned as a 9.6kb chromosomal fragment from *Bacillus stearothermophilus* strain BR388 into *Escherichia coli*. It conferred growth on limonene as a sole carbon source and bioproduction of α-terpineol, perillyl alcohol, and perillyl aldehyde. Incubation of the recombinant with perillyl alcohol resulted in formation of perillyl aldehyde and perillic acid. Catabolic pathways apparently affected the microbial toxicity of limonene.

Key Words: cloning, limonene degradation, *E. coli, Bacillus stearothermophilus*

INTRODUCTION

BECAUSE OF ITS LOW COST and extensive availability from citrus waste, the monoterpene limonene ((4R)-(+)-4-isopropenyl-1-methylcyclohexene) is a practical starting material for bioconversions to higher value flavor and fragrance constituents (Braddock and Cadwallader, 1992). Studies have indicated the ability of some microorganisms to convert limonene to specialty chemicals of interest including α-terpineol, carvone, and perillyl aldehyde and alcohol (see Krasnobajew, 1984; Kieslick et al, 1986). Progress towards bioproduction of monoterpene products from limonene has been impeded by the multiplicity and microbial toxicity of limonene metabolites (see Dhavalikar et al, 1966; Uribe and Pena, 1990; Chadstein et al, 1992). We initiated an examination of limonene pathways in aerobic thermophiles in the expectation that thermostable enzymes of such organisms may provide resistance to chemical inactivation by limonene (for discussion of enzyme chemical and thermostability, see Tombs, 1985). Microbial catabolic pathways have not been elucidated for monoterpenes in mesophilic or thermophilic microorganisms. Cloning of the limonene pathway would enable better understanding of the enzymes participating in monoterpene catabolism and the biochemical origin of observed metabolites. This may lead to controlling their formation in biotechnological applications. We described isolation and characterization of a *Bacillus stearothermophilus* thermophile (Chang and Oriel, 1994), capable of limonene degradation with production of α-terpineol and perillyl alcohol. Our current objective was to describe the cloning of the functioning limonene pathway from this thermophile into *E. coli*, and some physiological characteristics of the recombinant.

MATERIALS & METHODS

Bacterial strains, plasmids, media and reagents

Bacillus stearothermophilus BR388 described (Chang and Oriel, 1994), was grown and maintained on DP minimal medium with limonene vapor or LB medium at 55°C. *Escherichia coli* XL-1 [*recA-(recA1, lac-, endA1, gyrA96, thi, hsdR17, supE44, relA1,* F' *proAB, lacI$_q$, lacZDM15, Tn10*)] (Bullock et al., 1987), used for construction and maintenance of plasmids, was cultured at 37°C on LB medium. When appropriate, the medium was supplemented with ampicillin (50 μg/mL), tetracycline (12.5 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-galactoside (40 μg/mL). pBluescriptII SK$^-$ (Stratagene, La Jolla, CA) was used as a cloning vector. Plasmids were introduced into *E. coli* by electroporation using *E. coli* XL-1 cells in exponential phase washed in distilled water and resuspended in 10% glycerol in water. The electroporation procedure utilized a 0.2 cm gap cell with the following conditions: voltage, 2.5 KV; capacitance, 25 mF; and pulse control, 200 Ohms (Dower et al., 1988). The monoterpenes (+) limonene, (+) perillic acid, and α-terpineol were obtained from Aldrich Co. (+) perillyl alcohol and (+) perillyl aldehyde were purchased from Nippon Terpene Chemical Co., Japan.

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DNA procedures

Plasmid DNA was isolated from *E. coli* by the alkaline lysis method (Birnboim and Doly, 1979). Total DNA of *B. stearothermophilus* BR388 was prepared by the method of Saito and Miura (1963). Following partial restriction digestion with EcoRI restriction enzyme, BR388 DNA fragments with sizes > ≈5 kb were isolated from agarose gels by electroelution (IBI, New Haven, CT) and alkaline phosphatase-treated prior to ligation into the vector pBluescriptII SK$^-$ (Stratagene, La Jolla, CA) which was also cleaved with EcoRI. Restriction enzymes, DNA ligase, and alkaline phosphatase were purchased from Boehringer Mannheim Co., Indianapolis, IN, and utilized as suggested by the manufacturer. For hybridization studies, The 9.6 kb BR388 cloned fragment conferring growth on limonene when expressed in *E. coli* was labeled by random-primed incorporation of digoxigenin (DIG)-labeled deoxyuridine-triphosphate (dUTP) and detected using a DIG DNA labeling and detection kit, (Boehringer Mannheim Co., Indianapolis, IN). Hybridization was carried out using conditions recommended by the manufacturer using procedures described by Maniatis et al. (1989).

Screening for transformants capable of limonene growth

Transformants were spread on Petri plates containing M9 salt agar medium (pH 7.4, containing/L. Na_2HPO_4, 6g; KH_2PO_4, 3g; NaCl, 0.5g; NH_4Cl, 1g; Bactoagar (Difco, Inc.), 20g; followed after autoclaving with addition of 2 mL 1M $MgSO_4$, Maniatis et al., 1989) supplemented with 50 μg/mL of ampicillin and 40 μg/mL of X-gal containing 100 μL of limonene in a small glass tube attached to the plate cover, and incubated at 37°C for 6 days or longer. Cultures demonstrating growth on repeated transfer were selected for further examination.

Growth and biotransformation studies

Triple-baffled 250 mL culture flasks with a side arm (Bellco Glass Inc., Vineland, NJ) were used for microbial growth and biotransformation studies. These flasks allowed vapor introduction of (+) limonene, (+) perillyl alcohol, or α-terpineol into the culture from terpene contained in the side arm.

For perillyl alcohol biotransformation studies, a 1% inoculum of cells grown in M9 medium on limonene vapor with 0.006% yeast extract (OD580 of 0.08) was introduced into 75 mL M9 salts medium with 0.006% yeast extract and perillyl alcohol contained in the sidearm of a 250 mL closed culture flask. After 36h incubation with shaking at 37°C, cells were removed by centrifugation and terpene products in the supernatant analyzed.

Product extraction and analysis

After cell removal by centrifugation, the culture supernatant was separated into acidic and neutral fractions and extracted with ether as described (Chang and Oriel, 1994). The GC-MS system utilized was a

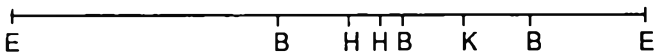

Fig. 1—Partial restriction map of the 9.6kb plasmid insert in EC409A. Abbreviations: E, EcoRI; B, BamHI; H, HindIII; K, KpnI. No cleavage sites were detected within the insert for restriction enzymes ClaI, PstI, SalI, and XbaI.

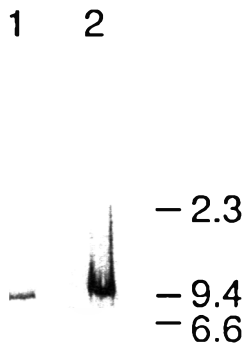

Fig. 2—Southern hybridization using the EC409A cloned insert from BR388 as a probe. Molecular weight marker positions are noted. Lane 1: EcoRI digest of BR388 DNA. Lane 2, EcoRI digest of recombinant plasmid of EC409A.

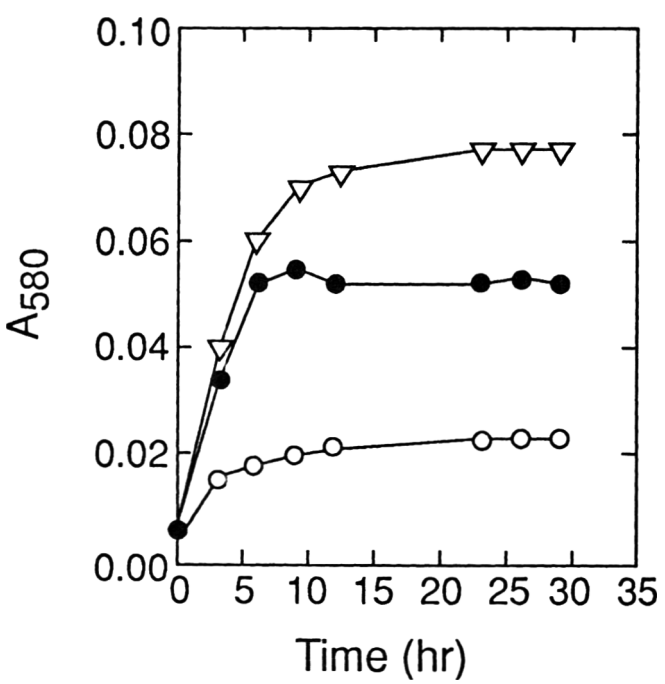

Fig. 3—Growth of *E. coli* recombinant EC409A on M9 salts supplemented with limonene and/or yeast extract. (○), limonene alone; (●), 0.006% yeast extract alone; (△), 0.006% yeast extract and limonene vapor.

Table 1—Strains used[a]

Strain	Description	Reference
BR388	*B. stearothermophilus* isolate, lim+	Chang and Oriel, 1994
XL-1	*E. coli* host strain, tc+, lim−	Bullock et al, 1987
EC409A	*E. coli* XL-1 with 9.6 kb BR388 DNA insert in pBluescriptII vector. tc+, lim+	This work

[a] Abbreviations: tc+, tetracycline resistant; lim+, grows on limonene as sole carbon source.

Table 2—Limonene bioconversion products of *E. coli* transformant EC409A

Growth stage	Metabolites	Concentration (mg/L)
early log (6h)	α-terpineol	1.4
late log (24h)	α-terpineol	33
	perillyl alcohol	0.51
	perillyl aldehyde	trace
	bicyclic monoterpene	trace
stationary (48h)	α-terpineol	1.0

Table 3—Bioconversion of perillyl alcohol by *E. coli* EC409A

Metabolite	Concentration, mg/L
perillyl aldehyde	36
perillic acid	230
α-terpineol	not detectable

HP5980 gas chromatography (Hewlett Packard, Farmington, NY) coupled with a HP5970 mass spectrometer. The mass detector was an MSD HP5970 (Hewlett Packard, Farmington, NY). A 0.25 mm ID × 30 m DB-wax fused silica capillary column (J & W Scientific Co., Folson, CA) was used for separation. Running conditions were 1 µL injection; He carrier gas; injection port and detector port at 240°C; column programmed from 40 to 240°C at 7°C/min with a 2 min initial hold time. Compounds were identified using comparisons of mass spectra and retention times with authentic monoterpene standards.

RESULTS

Cloning of the limonene pathway

E. coli XL-1 was transformed with pBluescriptII SK⁻ vector containing BR388 chromosomal DNA inserts. After growth on M9 salts agar plates containing ampicillin and incubation with limonene vapor, small colonies (designated lim+) were observed at a low frequency after 6 days. Growth on limonene vapor was not observed with untransformed *E. coli* XL1. Following verification of growth on limonene vapor by subsequent transfers, one lim+ transformant, designated EC409A, was selected for further characterization. It contained a 9.6 kb insert with the restriction map shown in Fig. 1. Hybridization experiments (Fig. 2) indicated a single copy of this insert was in the chromosome of the BR388 thermophile parent.

Growth studies of *E. coli* EC409A in liquid culture

Growth studies of transformant EC409A in M9 salts with limonene vapor showed that the transformant could utilize limonene vapor as sole carbon source in liquid cultures, although biomass levels were low (Fig. 3). As expected, no growth of EC409A was observed in M9 salts in the absence of limonene. Addition of yeast extract at low concentration (60 mg/L) elevated biomass levels while retaining limonene-stimulated growth and was used in other experiments.

Production of monoterpenes during growth on limonene

Monoterpene products were produced by recombinant EC409A during growth on limonene vapor and 60 mg/L yeast extract (Table 1). α-Terpineol was a major product, with highest

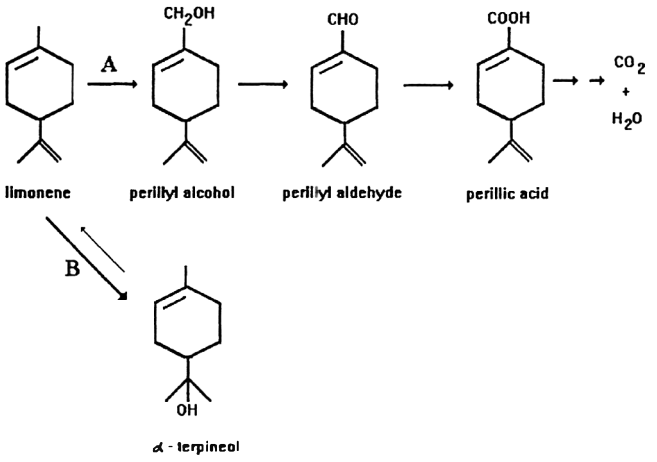

Fig. 4—Proposed limonene degradation pathway for *B. stearothermophilus* BR388 and *E. coli* EC409A. B denotes limonene conversion to dead end metabolite α-terpineol, and A denotes suggested main pathway for limonene catabolism.

production during exponential growth and decreased amounts in the stationary phase. Perillyl alcohol and perillyl aldehyde were also observed during the exponential phase.

Growth on and bioconversion of other monoterpene

When cells grown to exponential phase on limonene were resuspended in M9 salts with perillyl alcohol vapor, notable amounts of perillaldehyde and perillic acid were produced after 36 hr (Table 2). In separate experiments using terpenes as sole carbon source, no growth was observed on either perillyl alcohol or α-terpineol as sole carbon source.

DISCUSSION

WE BELIEVE THESE STUDIES constitute the first reported cloning of a functional microbial monoterpene degradation pathway. Consistent with parallel studies with a *B. stearothermophilus* benzene/toluene pathway, (Natarajan and Oriel, 1994), the *B. stearothermophilus* limonene pathway was functional when introduced into *E. coli*, enabling growth on limonene as a sole carbon source. Various limonene oxidation products of different microrganisms have been reported resulting in a several proposed pathways for limonene degradation (see Krasnobajew, 1984). The demonstrated production of both α-terpineol and perillyl alcohol by the EC409A recombinant should help determine which of the observed metabolites contribute to cellular energy. Further research is needed to firmly establish the growth-supporting pathway. However, the facile conversion of perillyl alcohol to perillyl aldehyde and perillic acid by the recombinant suggests participation of perillyl derivatives (Fig. 4).

Availability of this *E. coli* recombinant should allow elucidation of participating limonene pathway enzymes and metabolites. However, levels of desired monoterpene products produced are not sufficiently high to be of commercial interest. This may be due to further catabolism of limonene products by the recombinant. Also some microbial toxicity of limonene or its metabolites may remain as evidenced by low biomass levels. Successful limonene bioconversions will require further understanding and control of participating pathway enzymes in the producing microorganism. Possible employment of strategies such as adsorbent beads or two-phase systems may allow limonene introduction and monoterpene product removal during bioproduction. The recombinant we describe should prove useful in testing the utility of such approaches.

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Microbial Biosensor System for Rapid Determination of Vitamin B_6

HIDEAKI ENDO, ATSUSHI KAMATA, MASAKAZU HOSHI, TETSUHITO HAYASHI, and ETSUO WATANABE

ABSTRACT

A microbial biosensor system was based on an immobilized microorganism (*Saccharomyces uvarum* 4288 ATCC9080) and Clark-type oxygen electrode consisting of a platinum cathode, lead anode, alkaline electrolyte, and an oxygen permeable Teflon membrane. The determination was based on the respiratory activity of the microorganism in presence of vitamin B_6. Optimum conditions were: concentration of immobilized cells on membrane: OD_{660} = 0.05, temperature: 30°C. When oxygen permeable Teflon membranes (0.5 mil and 2.0 mil) were employed, relationships between vitamin B_6 (pyridoxine) and current decrease were linear in the range of 0.5 ng/mL–2.5 ng/mL and 2.5 ng/mL–12.5 ng/mL, respectively. One assay could be completed within 15 min. This system provided rapid and simple determinations of vitamin B_6 in marine products with good correlation to traditional microbial assay values.

Key Words: microbial sensor, biosensor, vitamin B_6, immobilized cells

INTRODUCTION

VITAMIN B_6 can be classified into pyridoxine (PIN), pyridoxal (PAL), pyridoxamine (PAM) and their phosphoric ester compounds pyridoxine phosphate (PIN-P), pyridoxal phosphate (PAL-P) and pyridoxamine phosphate (PAM-P). To determine vitamin B_6 in foods, several methods such as microbial (Rabinowotz et al., 1947; Fukui et al., 1953), enzymatic (Wada et al., 1957) and HPLC (high performance liquid chromatography) (Morita and Mizuno, 1980; Vanderslice et al., 1981) assays have been used. The microbial method is most widely used among the established methods. It is based on growth rate of a microorganism (*Saccharomyces carlsbergensis*) in presence of vitamin B_6, and shows good sensitivity and reproducibility. However, it requires incubation of the microorganism for >16 hr. A more rapid and accurate method for determination of vitamin B_6 is needed.

Various biosensor systems including microbial and enzyme sensors, consisting of immobilized biocatalysts and electrochemical devices, have been developed for determination of vitamins (Matsunaga et al., 1978; Karube et al., 1987; Endo et al., 1994). Matsunaga et al. (1978) developed a microbial sensor system for determination of vitamin B_1 by using *Lactobacillus fermenti* and oxygen electrode. A microbial sensor reported by Karube et al. (1987) measured vitamin B_{12} using *Escherichia coli* 215 and oxygen electrode. These sensor systems provided rapid and simple analyses. For food analysis, we developed an enzyme sensor system for determination of vitamin C (Endo et al., 1994) as well as other biosensor systems for measurement of glucose (Watanabe et al., 1986), fish freshness (Watanabe et al., 1987; Li et al., 1992) and phosphate ion (Watanabe et al., 1988).

Our current objective was to develop a microbial biosensor system for the rapid determination of vitamin B_6. The operational conditions of the sensor system were investigated and then the system was applied to determine vitamin B_6 in marine products.

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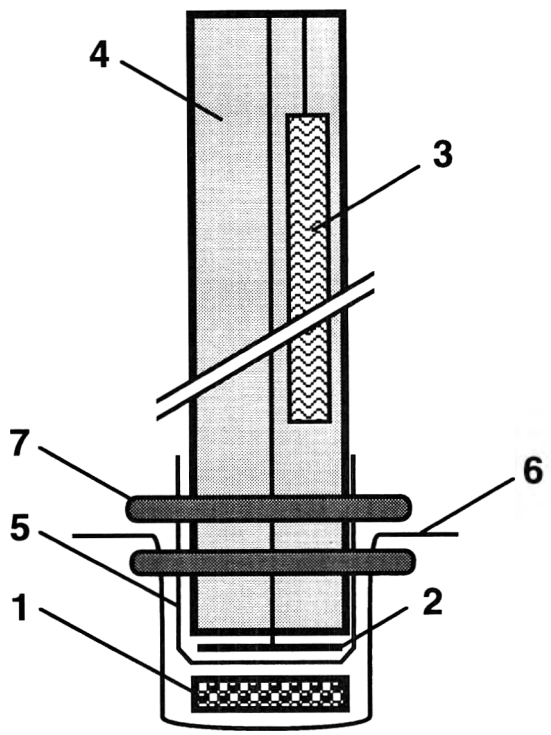

Fig. 1—Diagram of microbial electrode. (1) membrane containing immobilized microorganism; (2) Pb (cathode); (3) Pb (anode); (4) KOH; (5) oxygen permeable Teflon membrane (thickness: 0.5 mil or 2.0 mil); (6) dialysis membrane; (7) rubber ring.

MATERIALS & METHODS

Materials

Reagents. PIN was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). PAL and PAM were obtained from Sigma (Missouri USA). Vitamin B_6 Assay Medium Base (VAMB) was purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). The composition of the medium (1L) was as follows: casamino acid, 8g; inositol, 50 mg; thiamine hydrochloride, 500 µg; nicotinic acid, 5 mg; calcium pantothenate, 5 mg biotin, 16 µg; KCl, 850 mg; glucose, 100g; $CaCl_2$, 250 mg; $MgSO_4$, 250 mg; $MnSO_4$, 5 mg; KH_2PO_4, 1.1 g; iron (III) chloride, 5 mg; potassium citrate, 10 g; citric acid, 2 g. Peptone, malt extract and yeast extract were obtained from Difco laboratories (Michigan, USA). Dialysis membrane and oxygen permeable Teflon membrane were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Able Co. Ltd. (Tokyo, Japan).

Standard solution of vitamin B_6. PIN was used as a representative of vitamin B_6 complex. For preparation of stock solutions, 20 mg of PIN was dissolved in 100 mL of 25% ethanol aqueous solution. The stock solution was diluted with distilled water to prepare standard solutions in the range of 0.5 ng/mL–12.5 ng/mL at the beginning of each experiment.

Microorganisms and cultivation

Saccharomyces uvarum 4288 ATCC9080 was used as a biocatalyst of the microbial sensor. The microorganism was cultivated in Yeast and Mold Agar (YMA) which contained (g/L) yeast extract (3.0), malt extract (3.0), peptone (5.0), glucose (10.0) and agar (20.0), and incubated at 30°C for 16 hr.

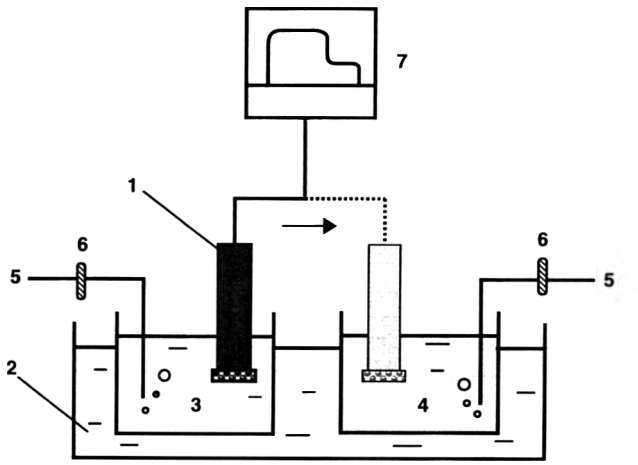

Fig. 2—Diagram of microbial biosensor system for the determination of vitamin B_6. (1) microbial electrode; (2) water bath; (3) vessel of 0.05M phosphate buffer; (4) vessel of VAMB solution; (5) air; (6) filter; (7) recorder.

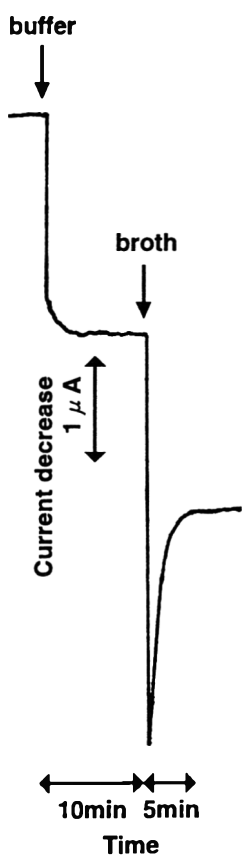

Fig. 3—Response curve of microbial biosensor system. Vitamin B_6 (PIN) concentration: 1.0 ng/mL, immobilized cell concentration: OD_{660} = 0.05, temperature: 30°C.

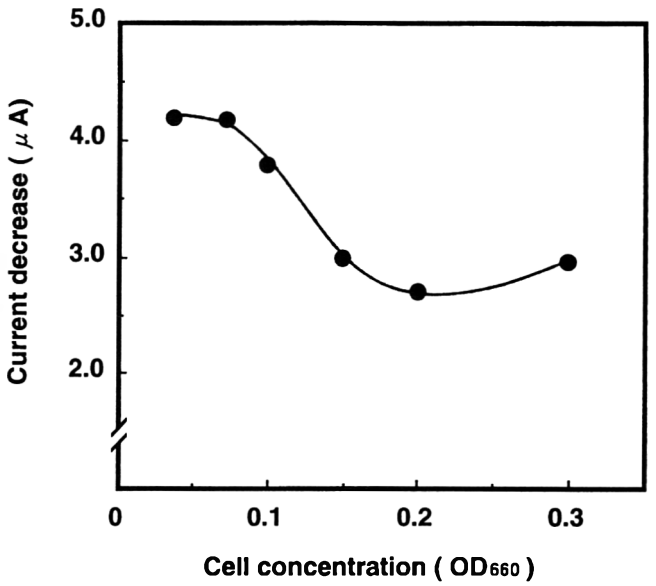

Fig. 4—Effect of immobilized cell concentration on the current decrease of the sensor. The experimental conditions same as in Fig. 3.

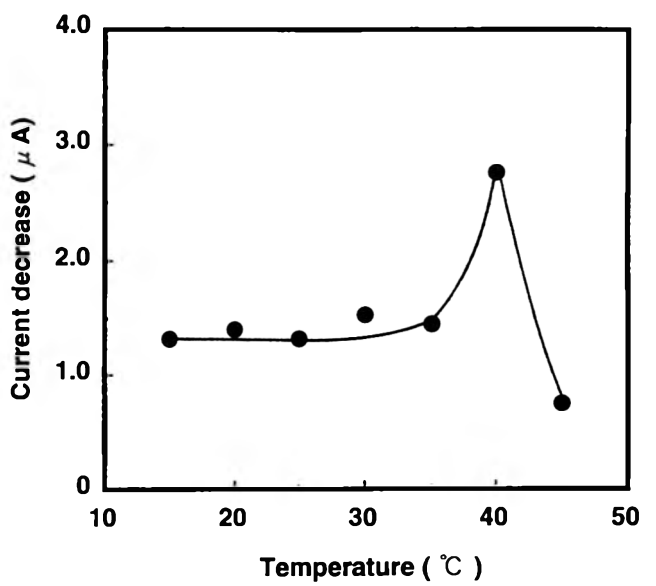

Fig. 5—Effect of temperature on the current decrease of the sensor. Other experimental conditions same as in Fig. 3.

Preparation of microbial electrode

One colony of *S. uvarum* 4288 ATCC9080 cultivated in YMA was suspended in 0.9% NaCl solution resulting in an optical density at 660 nm (OD_{660}) in the range 0.037–0.300. To prepare the membrane with immobilized cells, several sheets of cellulose nitrate membranes (pore size: 0.45 μm, diameter: 13 mm, Advantec Toyo Ltd. (Tokyo, Japan)) were sterilized with steam and the cell suspension (1 mL) was filtered through each membrane. Constant activity of each membrane with immobilized cells was obtained by storing at 4°C for ≥1 hr. One of the membranes was tightly set on a platinum cathode of Clark-type oxygen electrode (Able Co., Tokyo, Japan) and covered with a dialysis membrane. The oxygen electrode consisted of a platinum cathode (diameter: 11 mm), a lead anode, alkaline electrolyte (KOH), and an oxygen permeable Teflon membrane (thickness: 0.5 mil or 2.0 mil). Dialysis membrane was fixed on the tip of the electrode using a rubber ring (Fig. 1). The membrane with immobilized cells was replaced after each measurement with another membrane stored at 4°C.

Preparation of samples of marine products

Flatfish (*Limande yokohamae*), jack mackerel (*Trachurus japonicus*), sardine (*Sardinops melanostictus*), and kumura prawn (*Penaeus japonicus*) were used as sources of vitamin B_6 in marine products to evaluate effectiveness of the biosensor. To extract vitamin B_6 from marine products, 1 g of muscle or viscera was homogenized with 10 mL of ethanol solution (60%). The homogenate was shaken in a flask for 40 min at 200 rpm and centrifuged for 3 min at 4,000 rpm. After the supernatant of the sample was collected, the precipitate was resuspended with ethanol solution and centrifuged again. This was repeated three times. To prepare free PIN, PAL and PAM from corresponding phosphoric ester

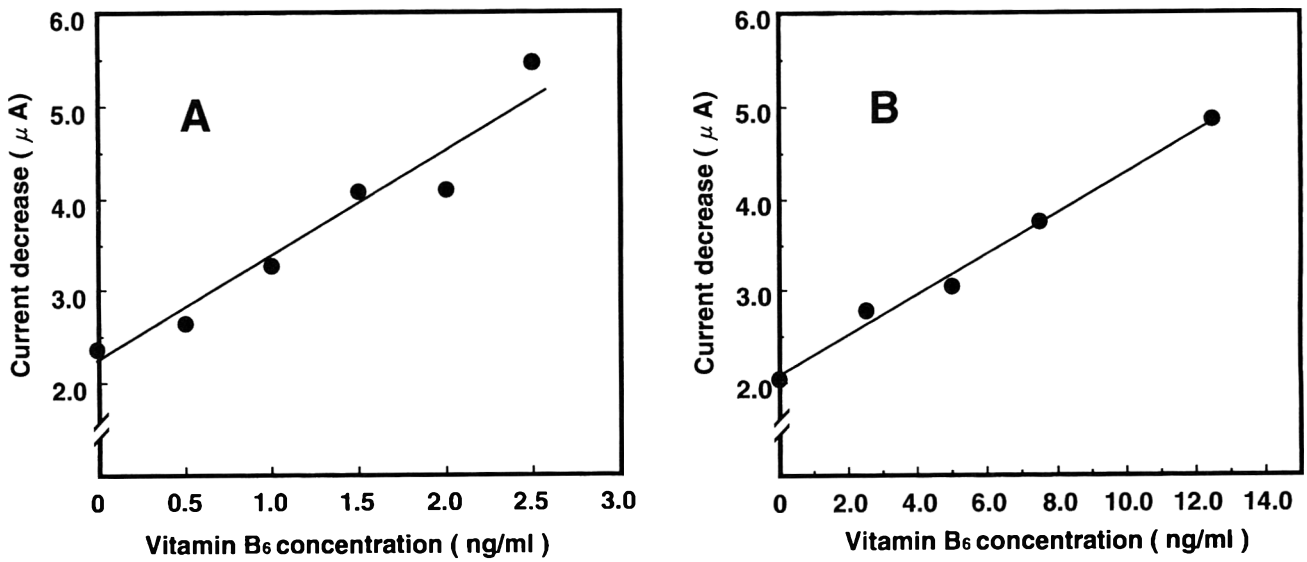

Fig. 6—Calibration curves for vitamin B_6 (PIN). Oxygen permeable Teflon membrane, thickness 0.5 mil (A) and 2.0 mil (B) were employed. Experimental conditions as in Fig. 3.

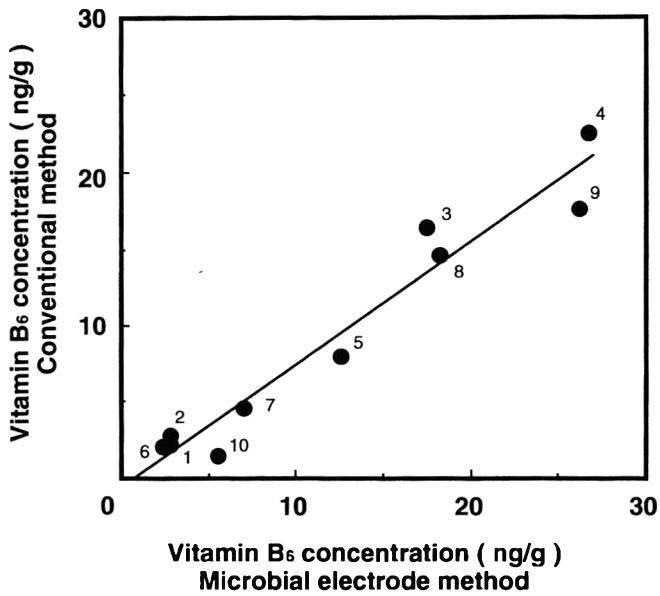

Fig. 7—Correlation between vitamin B_6 data determined by sensor and conventional methods. Flatfish (*Limande yokohamae*): (1) liver, (2) dark muscle, (3) dorsal muscle, (4) belly muscle; jack mackerel (*Trachurus japonicus*): (5) dark muscle, (6) liver; sardine (*Sardinops melanostictus*): (7) dorsal muscle, (8) dark muscle, (9) liver; kumura prawn (*Penaeus japonicus*): (10) belly muscle.

compounds, the ethanol extract dissolved in 0.1N H_2SO_4 was heated at 115°C for 4 hrs.

Apparatus and assay procedure

Microbial sensor method. This assay system consisted of a microbial sensor (Fig. 2) vessels of 0.05M phosphate buffer (15.0 mL, pH 6.0) and VAMB solution (144 g/L, 13.5 mL, pH 6.0), a water bath, and a recorder. Both phosphate buffer and VAMB solution were saturated with oxygen by bubbling air through the solutions. 1.5 mL of standard or sample solutions was added to the VAMB solution. To determine the concentration of vitamin B_6, the microbial sensor was immersed in the buffer vessel first. After the output current of the microbial sensor became stable, the sensor was removed and immediately placed in VAMB solution. When a stationary current was obtained, the current decrease was measured. Concentrations of vitamin B_6 could be calculated by the following formula:

$$[V\ B_6] = I/K$$

where [V B_6] = vitamin B_6 concentration (mM); I = current decrease of microbial sensor; K = slope of calibration curve for vitamin B_6.

Conventional method. The microbial assay was used as a conventional method. One platinum loop of *S. uvarum* 4288 ATCC9080 cultivated in YMA was suspended in 100 mL of 0.9% NaCl solution to prepare the inoculum. The cell suspension (0.5 mL) was inoculated to a test tube containing 1.5 mL of standard or sample solutions and 2.0 mL of VAMB solution (conc.: 260 g/L). The test tube was incubated at 30°C for 18 hr and OD_{660} of the culture broth was measured by a spectrophotometer (Type: UV-160, Shimadzu Co. Ltd., Tokyo, Japan). Vitamin B_6 concentration could be calculated from the calibration curve of the standard solution.

RESULTS & DISCUSSION

Response curve of microbial biosensor

When the microbial sensor was immersed in the buffer vessel, the output current of the sensor became stable within 10 min (Fig. 3). After steady current was obtained, the sensor was transferred to VAMB solution containing 5 ng of PIN. The output current began to decrease within 20 sec and the minimum current was observed within 30 sec. Then the current gradually increased again and reached a plateau within 4 min. The net current decrease was 1.7 μA.

These current changes indicated that vitamin B_6 and various compounds in VAMB solution had passed through the cellulose nitrate membrane and were assimilated by the immobilized microorganism. Oxygen consumption due to respiratory activity of the microorganism caused a decrease in dissolved oxygen around the membrane and consequently brought about the decrease in output current. The current gradually increased again because oxygen diffused continuously into the cellulose nitrate membrane from VAMB solution. When the assimilation rate of vitamin B_6 by the microorganisms and the diffusion rate of the dissolved oxygen attained equilibrium, the original and steady current was obtained. The difference in current decrease between the stationary current obtained from buffer solution and from VAMB solution was taken as the measure of vitamin B_6 concentration. One assay could be completed within 15 min.

The membrane with immobilized cells of biosensor was replaced with a new one for each measurement. The exchange of membrane was very easy by using a rubber ring (Fig. 1) and could be done in 3 min. The sensor thus prepared gave fairly

stable output current at each measurement (relative error: ±3%).

Effects of assay conditions on sensor response

In general, the response of the microbial biosensor was readily influenced by analytical conditions such as immobilized cell concentrations and temperature. Effects of these parameters on current decrease of the sensor were investigated further. The sensor response decreased with increasing cell concentration (Fig. 4). We assumed that the increase of immobilized cell concentration on the membrane influenced the respiratory activity of the microorganism, because the concentration of PIN was limited in the VAMB solution (1.0 ng/mL). The respiratory activity may have decreased due to depletion of PIN by increasing cell concentrations. Since a high response was observed in the range OD_{660} = 0.037–0.075, the immobilized cell concentration of the membrane was prepared in that range (OD_{660} = 0.05).

The response of the sensor was constant until 30°C (Fig. 5) but rapidly increased at about 35°C and decreased again at 45°C. Maximum response was obtained at 40°C. Higher response would be preferable for operation of the sensor. However, the response became unstable at 40°C because this system was based on measurement of dissolved oxygen concentration. Operation at 30°C was concluded as optimum for the system. Thus the sensor system was operated at the following optimum conditions; immobilized cell concentration OD_{660} = 0.05, temperature: 30°C.

Calibration curve for vitamin B_6

Oxygen permeable Teflon membrane with the thickness of 0.5 mil (Fig. 6A) and 2.0 mil (Fig. 6B) were employed. Calibration curves for (A) and (B) were linear in the range 0.5–2.5 ng/mL and 2.5–12.5 ng/mL, respectively. Sensitivity of the thinner sensor was higher because oxygen easily permeated through it. However, the calibration curve plateaued at >2.5 ng/mL PIN. The thicker sensor (B) was applicable in a wide range of vitamin B_6 concentrations although sensitivity was lower at <2.5 ng/mL PIN. Either sensor system (A) or (B) could be used depending on vitamin B_6 concentration of the sample.

The relationship between the current decrease of the sensor and the concentration of other vitamin B_6 compounds such as PAL and PAM was also investigated. When 0.5 mil thickness of oxygen permeable Teflon membrane was employed, calibration curves for PAL and PAM were linear in the range 1.0–2.5 ng/mL and 1.0–5.0 ng/mL, respectively.

Application for vitamin B_6 in marine products

The microbial biosensor was applied for determination of vitamin B_6 in marine products. Correlation between the proposed sensor method and the conventional method was determined using ethanol extracts of flatfish (*Limande yokohamae*), jack mackerel (*Trachurus japonicus*), sardine (*Sardinops melanosticus*), and kumura prawn (*Penaeus japonicus*). Various portions of each marine product such as liver, dark muscle, dorsal muscle, and belly muscle were examined. The concentration of vitamin B_6 was calculated from the equation described above. A high correlation was found between values determined by microbial sensor and the conventional method (Fig. 7, correlation coefficient = 0.95). Since one assay was completed within 15 min, this system could be used for rapid determination of vitamin B_6 in marine products. Further studies in our laboratory are developing microbial biosensor systems which can analyze phosphoric ester compounds such as PIN-P, PAL-P and PAM-P.

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Hypocholesterolemic Potential of Oat Bran Treated with an Endo-β-D-glucanase from *Bacillus subtilis*

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ABSTRACT

Oat bran was treated with endo-β-D-glucanases to hydrolyze β(1→3) (1→4)-glucans. Hydrolysis of β-glucans reduced the *in vitro* viscosity of a suspension of oat bran. Untreated oat bran and hydrolyzed oat bran were incorporated into hypercholesterolemic purified diets and fed to rats for 4 wk; a diet containing cellulose was used as a control. Hepatic cholesterol accumulation was affected by dietary treatment and was less in the oat bran group than in the enzyme-treated oat bran or cellulose groups. Thus, functional properties of (1→3) (1→4)-β-D-glucan, such as viscosity, appear to be related to its hypocholesterolemic potential.

Key Words: oat bran, endoglucanase, hypocholesterolemia, cholesterol

INTRODUCTION

OAT BRAN has been reported to lower plasma cholesterol in humans and animals (Kirby et al., 1981; Shinnick et al., 1990). A meta-analysis of oat bran studies in humans confirmed that reduction of plasma cholesterol was independent of modifications in fat or carbohydrate intake when diets were supplemented with oat bran. The higher the initial cholesterol concentration the greater was the reduction due to oat bran consumption (Ripsin et al., 1992). Physical properties such as viscosity, bile acid absorption capacity, and fermentability have been associated with the hypocholesterolemic response to dietary fibers (Eastwood and Morris, 1992).

The (1→3) (1→4)-β-D-glucan polysaccharide (β-glucan) found in oat bran is viscous, highly fermentable, and has been demonstrated to lower blood cholesterol in rats (Klopfenstein, 1988; Shinnick et al., 1991; Wood et al., 1989). Several studies in rats demonstrated that fiber supplements enriched in the oat β-glucan had enhanced cholesterol-lowering effects (Chen et al., 1981; Jennings et al., 1988; Klopfenstein and Hoseney, 1987). However, because animals consuming high β-glucan diets may gain less weight than control animals, comparison of cholesterol levels among groups is not directly valid. An alternative experimental approach would be the enzymatic hydrolysis of β-glucan by a purified endo-β-D-glucanase that cleaves the polysaccharide into oligomers (Kato and Nevins, 1984). Treatment of oat bran with such enzyme would directly reduce the viscosity of hydrated oat bran but all fragments of β-glucan breakdown would remain available for fermentation in the large intestine. Our objective was to evaluate the cholesterol-lowering potential of oat bran containing intact β-glucan as compared to oat bran containing β-glucan fragments.

MATERIALS & METHODS

Oat bran

Oat bran from a single lot was provided by the Quaker Oats Company (Barrington, IL). The cereal was divided and processed in duplicate batch treatments to yield enzyme-treated oat bran (ETOB) and oat bran subjected to all treatments but the enzyme step (OB). Dried cereal was hydrated (20 mL water/1g oat bran) and autoclaved (20 min at 100°C) to inactivate constitutive enzymes. The solutions were cooled to 40°C and buffered by adding sufficient concentrated Na_3PO_4 (pH 6.0) to achieve a final concentration 10 mmol/L. (1→3) (1→4)-β-D-glucanase was purified from a commercial *Bacillus subtilis* enzyme preparation (Novo Ban 120, Novo Nordisk Bioindustrials, Inc., Danbuy, CT) according to the procedure described by Kato and Nevins (1984). A single protein band was disclosed upon SDS-PAGE and, with a range of substrates, no other glucanase activities were detected. Sufficient glucanase was added to each ETOB sample (ca. 400 µg) to complete glucan degradation within 5 hr incubation based on data from preliminary experiments. Both the ETOB and OB were maintained at 40°C in a water bath with intermittent stirring. A few drops of toluene were added to suppress microbial activity. Batches were lyophilized to insure that no soluble products resulting from the treatment would be lost in sample recovery. Batches (3 kg each) were combined to provide a supply of ETOB and OB for feeding studies.

The appearance of reducing sugars (Somogyi, 1951) provided a basis to verify the progression of β-glucan hydrolysis during enzyme treatment. Aliquots of soluble products were centrifuged, filtered (0.45 micron mesh) and 20 µL samples injected on a HPX-42A column (Bio-Rad Laboratories, Hercules, CA) for HPLC analysis. Fractions were eluted with water (0.6 mL/min^{-1}) at 85°C and detected by refractive index to evaluate the extent of glucan depolymerization in both ETOB and OB.

Total dietary fiber (TDF) and insoluble dietary fiber (IDF) of ETOB and OB were analyzed by the AOAC method (Prosky, 1988). Soluble dietary fiber (SDF) was calculated by difference.

Rheological characterization of ETOB and OB was conducted on a Carri-Med Controlled Stress rheometer (Dorking, Surrey, UK) at 25°C. Initial measurements were conducted with hydrated samples immediately following the 5-hr enzyme treatment. Subsequent samples were rehydrated from the dried material in a dilution series (1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 g/30 mL water). The more dilute samples (1.0, 1.5, and 2.0g) were centrifuged (9000 × g, 4°C, 10 min) to separate the water-soluble fraction from large, insoluble particles. Supernatant (≈18 mL) was placed in concentric cylinders with a 500 micron gap. The concentrated samples (2.5, 3.0, and 3.5g) were analyzed as a whole food system, using parallel plates (4 cm diam) with a 1000 micron gap. The most concentrated sample represented the cereal-to-water ratio used to cook oat bran. The data were fitted to a power law model which often has been used to describe pseudoplastic flow.

Animals and diets

Thirty male Wistar rats (Hilltop Labs, Scottsdale, PA) average initial weight 125g, were fed a stock diet (Rat Chow, Purina Mills, St. Louis, MO) for 7 days. The animals were each placed in a wire-bottom cage and housed in a temperature (21–23°C), and light-controlled room (12 hr light-dark cycle) at ambient humidity. Following the initial 7-day period, 10 animals were randomly assigned to each of three test diets. Food intake was measured daily for a 3-day period during the second and fourth week, and body weights were monitored periodically throughout the 5-wk feeding.

Three fiber supplements (ETOB, OB, and cellulose (CL)) were added to an AIN-76 diet (AIN, 1977 and 1980) (Table 1). The diets provided protein (20%), carbohydrate (58.8%), fat (10%), and TDF (5%). Cholesterol (1.0%) and cholic acid (0.2%) were added to the diet to induce hypercholesterolemia (Shinnick et al., 1990).

At the end of the 5-wk study, animals were fasted overnight and injected with a dose of ketamine (50 mg/kg body wt.), rompum (5 mg/kg), and acepromazine (0.75 mg/kg). Blood samples were obtained by heart puncture with EDTA (1 mg/mL blood) to prevent coagulation. The small and large intestine were removed and weighed. Livers were removed, weighed, and frozen in liquid nitrogen.

Plasma and liver lipid analyses

Whole blood was centrifuged (1500 × g, 4°C, 20 min) to obtain plasma, which was stored at −20°C until analyzed. Partially thawed liver

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Table 1—Diet composition (g/kg)

	Diet	
	CL	OB or ETOB[a]
Cellulose	50.0	0
OB or ETOB	0	370.0
Casein	200.0	127.0
Cornstarch	300.0	136.0
Sucrose	288.0	266.0
Corn oil	100.0	72.0
Mineral mix[b]	35.0	35.0
Vitamin mix[c]	10.0	10.0
Choline chloride	2.0	2.0
DL-Methionine	3.0	3.0
Cholesterol	10.0	10.0
Cholic acid	2.0	2.0
BHT	0.2	0.2

[a] OB = oat bran, ETOB = enzyme treated oat bran.

[b] Mineral mix (g/kg): $CaCO_3$, 18.0; K_2HPO_4, 19.5; $CaHOP_4$, 3.6; NaCl, 10.08; $FeSO_4$-$7H_2O$, 1.5; $MgSO_4$-H_2O, 3.88; KI, 0.015; $ZnCO_3$, 0.048; $CuSO_4$-$5H_2O$, 0.018; $MnSO_4$-H_2O, 0.138.

[c] Vitamin mix (g/kg): Choline chloride, 50.0; myo-inositol, 25.0; ascorbic acid, 5.0; calcium pantothenate, 2.5; thiamin hydrochloride, 1.5; pyridoxine, 1.5; nicotinic acid, 1.5; menadione, 1.25; riboflavin, 0.5; para-amino benzoic acid, 0.5; folic acid, 0.03; biotin, 0.125; reinyl palmitate, 2.1 (325,000 IU/g); Cholecalciferol, 0.23 (325,000 IU/g); B-12 + mannitol, 1.5; alpha-tocopheryl acetate, 21.4 (25,000 IU/g); cerelose, 885.

Table 2—Viscosity of OB and ETOB dispersions as related to concentration

Concentration (g/30 mL)	OB	ETOB
	cP[b]	
1.0[c]	6.0	3.0
1.5[c]	14.5	3.5
2.0[c]	22.0	4.5
2.5[d]	300	25
3.0[d]	550	125
3.5[d]	700	150

[a] OB = oat bran, ETOB = enzyme treated oat bran

[b] Viscosities in centiPoise (cP) were determined for a shear rate of 20s $^{-1}$

[c] Samples measured in concentric cylinders.

[d] Samples measured between parallel plates.

samples (≈0.5g) were used for lipid extraction with a modified Folch method (Folch et al., 1957). Total cholesterol was measured in plasma and liver extract using the cholesterol oxidase method (Allain et al., 1974). Plasma and liver free-cholesterol were also measured, with the omission of cholesterol esterase; esterified cholesterol was calculated as the difference between total and free cholesterol. Triglycerides were measured in plasma by estimated glycerol release (Sigma Catalog No. 336-50, St. Louis, MO) and concentration calculated on the basis of a triglyceride calibrator (Sigma Catalog No. T2772, St. Louis, MO). Liver triglycerides were measured with a colorimetric method, following purification and saponification of the lipid extract (Sigma, 1982).

Statistical analysis

The data were analyzed with one-way analysis of variance with least square difference to detect mean differences among the three groups using Statview II (Abacus Concepts, Inc., Berkeley, CA). Values were expressed as means ± SEM of each group. Values which differed significantly ($p < 0.05$) were identified with different superscript letters.

RESULTS

Oat bran characterization

TDF content of OB and ETOB was 15.0 and 13.3% (dry weight basis), and the IDF was 8.0% and 6.6%, respectively. The IDF of both OB and ETOB was about half of TDF. Thus, distributions of IDF and SDF fractions were similar for OB and ETOB.

Glucanase treatment of OB resulted in an increase in reducing sugar equivalents during the first 3 hr of enzyme treatment. The quantity of reducing groups in the control did not change during incubation while in ETOB the presence of the enzyme increased reducing groups at least fivefold. HPLC revealed a progressive increase in trisaccharides and tetrasaccharides accompanying the increase in reducing equivalents. Based on previous analysis of OB hydrolysis products we tentatively identified the trisaccharide as 3-0-cellobiosyl-D-glucase and the tetrasaccharide as 3-0-cellotriosyl-D-glucose. These characteristic oligosaccharides and the appearance of lesser amounts of additional oligosaccharides with higher molecular weights was consistent with the β-glucanase fragmentation pattern of (1→3) (1→4)-β-D-glucan (Kato and Nevins, 1984).

Preliminary rheology measurements with the parallel plates indicated that hydrated samples of ETOB were less viscous than OB. At an applied shear stress of 20 dynes/cm^2, the ETOB preparation had a viscosity of 2.8 cP, compared with the OB, 4.4 cP. This represented a 35% decrease in apparent viscosity of the hydrated oat bran preparation which had the enzyme treatment. The viscosity coefficient and rate index values from measurement with concentrated and dilute sample series were used to calculate shear stress levels using the power law model and an arbitrary shear rate of 20/sec (Barnes et al., 1989). The calculated viscosities were compared for the two dilution series (Table 2). OB exhibited a resistance to flow which was about twice that of ETOB at the most dilute concentration (1 g/30 mL) and nearly fivefold greater for the most concentrated sample (3.5 g/30 mL). That sample represented the cereal-to-water ratio used in the traditional preparation of cooked oat bran cereal. Increasing concentration resulted in a consistently greater resistance to flow for OB than for ETOB. Thus, hydrolysis of the β-glucan reduced the overall viscous nature of hydrated OB.

Table 3—Food intake and body weight

Diet	Cellulose	Oat bran	Enzyme-treated oat bran	Pooled SEM
Daily food intake, g				
2 wk	23.1[b]	20.8[a]	23.0[b]	0.7
4 wk	25.3[b]	21.7[a]	21.6[a]	0.8
Body weight, g				
Initial	218	223	228	6
2 wk	322[b]	287[a]	308[ab]	7
5 wk	436	401	414	11
Organ weights				
g/100g body wt				
Small intestine	1.76[a]	2.04[b]	1.83[a]	0.06
Large intestine	0.85	1.38	1.39	0.21
Liver	5.86[c]	4.72[a]	5.09[b]	0.13

[a,b] Values are means ± SEM. Values in a row with significant differences ($p < 0.05$) are denoted by different superscripts.

Animal study

Mean daily food intakes, body weights, and organ weights were compared (Table 3). No significant differences occurred in initial body weights among the three groups. At 2 wk, the daily feed intake of the OB group was less than ETOB or CL, and body weights of the OB group were less than that of CL, but not of ETOB. At 4 wk, the daily intake of both OB and ETOB groups was less than CL. At the completion of the feeding study, OB and ETOB groups weights were not significantly ($p = 0.10$) different. The mean weight of the small intestine of the OB groups was higher than that of the CL and ETOB groups. Differences in large intestine weight were not significant. Liver weight was different among all three groups with OB < ETOB < CL.

The cholesterol and triglyceride concentrations of plasma and liver (Table 4) showed plasma cholesterol did not differ between OB and CL groups but was higher in the ETOB group than OB and CL groups. The fraction of esterified cholesterol was 0.96 (pooled SEM = 0.002) in all three groups. Total liver cholesterol was different among the three groups, with OB < ETOB < CL. The fraction of esterified cholesterol in the liver was lower in the ETOB group than CL but the OB group did not differ from CL or ETOB.

Table 4—Plasma and liver cholesterol and triglyceride concentrations

	Cellulose	Oat bran	Enzyme-treated Oat bran	Pooled SEM
Plasma		mmol/L		
Cholesterol	2.20[a]	2.04[a]	2.70[b]	0.15
Triglycerides	1.02	0.83	0.89	0.11
Liver		mmol/g		
Cholesterol	1.47[c]	0.90[a]	1.21[b]	0.08
% Esterified[d]	0.90[b]	0.86[ab]	0.85[a]	0.01
Triglycerides	0.47[b]	0.31[a]	0.39[ab]	0.04

[a-c] Values are means, n = 10 per group. Values with significant differences ($p < 0.05$) are denoted by different superscripts.
[d] Fraction of esterified cholesterol, mg/mg.

Some differences in pattern of response between plasma and liver were observed. Thus, a value for cholesterol pool in these two tissues was estimated from the plasma plus liver cholesterol in the following manner: [(plasma cholesterol) × (0.03 × body weight) + (mg/g liver cholesterol) (g liver)]. The calculation estimated the total amount of cholesterol in each tissue. This value (in grams) was 1.55, 0.77, and 1.12 (pooled SEM 0.001) for the CL, OB, and ETOB groups, respectively, and was different ($p < 0.0001$) among the three groups (OB < ETOB < CL).

No significant differences occurred in plasma triglycerides among the three groups. The liver triglycerides of the OB group were lower than the CL group, but not different from ETOB. ETOB liver triglycerides were not different from OB or CL.

DISCUSSION

OUR RESULTS INDICATE that in rats fed a hypercholesterolemic diet, the hepatic cholesterol accumulation was lower in those fed OB containing the native β-glucan than in those fed OB with hydrolyzed β-glucan. Supplementation of the diet with 1.0% cholesterol and 0.2% cholic acid was effective in inducing elevated plasma and liver cholesterol levels in the rat. The cholesterol-lowering effectiveness of a fiber-supplement could be demonstrated by the ability to diminish such accumulations. Using this model the cholesterol-lowering effects of oat bran, pectin, psyllium, guar gum, and oat gum have been reported. However, the reductions in cholesterol were consistently greater for liver than for serum (Shinnick et al., 1990).

Values for TDF, IDF, and SDF were comparable between OB and ETOB. By hydrolyzing the β-glucan *in vitro*, the presence of soluble, fermentable polysaccharide and oligosaccharide products was retained while reducing the viscosity that was associated with the intact β-glucan. The TDF value of OB was 15.0%; this agreed with other published values. The USDA Human Nutrition Information Service reported the TDF of oat bran to be 15.9% (HNIS 1988). Anderson and Bridges (1988), using a modified version of the Englyst gas chromatography method, reported a value of 15.7% for TDF, 50% of which was measured as soluble fiber. We estimated that the soluble fiber content by difference of OB, and ETOB, was 7.0% and 6.6% respectively. Thus, the distribution of insoluble and soluble fiber was unchanged by the enzymatic treatment and the difference in the soluble fiber content of OB and ETOB was 1%. Although they had common properties, OB and ETOB had different effects on hepatic cholesterol concentrations and the calculated pool of plasma and hepatic cholesterol.

Both the intact β-glucan of untreated OB and the hydrolyzed oligosaccharides produced during glucanase digestion of ETOB are resistant to digestion in the mammalian small intestine but are susceptible to microbial degradation in the colon. The fermentation of glucan fragments would not likely be different from the native polymer. However, the glucan fragments might be more readily accessible to colon microflora. The similarity in large intestine weight, which includes contents, between the OB and ETOB group indicates that these diets provided a comparable amount of bulk for the large intestine. Therefore a primary difference between OB and ETOB was the capacity to increase viscosity. *In vitro* measurements demonstrated that hydrated OB was consistently more viscous than hydrated ETOB, and the increase in viscosity as the cereal-to-water ratio was increased was greater for OB than for ETOB. Such differences in flow properties *in vitro* are likely to influence the viscosity *in vitro* of the gut contents. The slight elevation in small intestinal weight was undoubtedly associated with the higher viscosity of OB, since compared to cellulose, OB has been reported to increase small intestinal length and the thickness of the smooth muscle layer (Schneeman and Richter, 1993). These results demonstrate that the greater cholesterol accumulation in the group fed ETOB compared to OB was related to reduction in viscosity of β-glucan. Others have reported that β-glucanase added to barley or rye feed increased weight gain and diminished the hypocholesterolemic response in the chick model (Bedford and Classen, 1992; Fadel et al., 1987; Pettersson and Aman, 1992). However, other factors may be associated with the β-glucan effect on cholesterol metabolism since liver cholesterol was lower in ETOB animals than in CL animals. The difference between ETOB and CL groups suggest that the oligomers may retain some hypocholesterolemic potential or that other components of oat bran may affect the response.

The TDF and SDF content of ETOB and OB were similar, but their effects on cholesterol metabolism were different. Therefore, the measurement of these fiber components may not be predictive of cholesterol-lowering potential of foods. Other factors, such as fiber molecular weight, conformation, or functional properties, are likely to be more important than soluble fiber content in predicting the hypocholesterolemic effect of OB.

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Cholesterol Removal from a Lard-water Mixture with β-Cyclodextrin

GOW-CHIN YEN and LI-JU TSAI

ABSTRACT

The effect on removing cholesterol from a lard-water mixture by β-cyclodextrin (β-CD) increased with increasing level of β-CD (1–10%). About 90% of cholesterol was removed from lard by 5% β-CD with 1:1 ratio of lard-to-water and stirring at 150 rpm and 27°C for 1 hr. The acid value of β-CD treated lard was significantly decreased due to removal of free fatty acid. The peroxide value of β-CD treated lard was slightly increased whereas the fatty acid composition apparently was not changed.

Key Words: cholesterol, lard, β-cyclodextrin, fatty acid

INTRODUCTION

THE RELATIONSHIP between dietary cholesterol and total serum cholesterol has been extensively investigated and suggestions have been made that dietary cholesterol contributes a risk factor in the development of coronary heart disease (Grundy et al., 1982). The content of plasma cholesterol has been well correlated to the dietary cholesterol. Furthermore, some cholesterol oxidation products have been reported as cytotoxic, atherogenic, mutagenic and carcinogenic (Maerker, 1987). Therefore, a lower intake of high cholesterol foods has been suggested as an effective method for lowering the level of serum cholesterol.

Various physical, chemical and biological methods have been proposed for reducing cholesterol in foods. These include blending with vegetable oils (Reiser, 1969; Durkley, 1982), extraction with organic solvent (Larsen and Froning, 1981), adsorption with saponin to form cholesterol complexes (Micich, 1990), vacuum distillation (Anon, 1982) and molecular distillation (Arul et al., 1988). In addition, the degradation of cholesterol by cholesterol oxidases (Watanabe et al., 1989a,b) and removal of cholesterol by supercritical carbon dioxide (Arul et al., 1988; McLachlan et al., 1990; Ong et al., 1990) have also been reported. However, most of these physical and chemical methods tend to be relatively nonselective, removing flavor and nutritional components with cholesterol. Moreover, some methods are high in operation cost.

β-Cyclodextrin (β-CD) is a cyclic oligosaccharide composed of α-(1, 4)-linkages of seven glucose unit members. β-CD has a cavity in the center of the molecule and has the capability of forming an inclusion complex with various compounds, including cholesterol (Szejtli, 1982a, b). Vollbrecht (1991) indicated that β-CD had high selectivity on cholesterol in its removal from egg yolk. β-CD also has advantages of non-toxicity, edibility, non-hygroscopicity, chemical stability and easy separation (Nagamoto, 1985). Thus, it may be a suitable substance for cholesterol removal from foods.

Edible commercial lard, used for hardening agents, shortening, cocoa butter substitutes and cooking oils, contains a high levels of cholesterol (ca. 50–120 mg/100g) (Courregelongue and Maffrand, 1989). Several studies have been reported on removal of cholesterol from animal fats with β-CD (Courregelongue and Maffrand, 1989; Davidson, 1990; Oakenfull and Sidhu, 1990; Makoto et al., 1992), but little information is available on cholesterol removal from lard by β-CD. Our primary objectives involved investigating the conditions for removal of cholesterol from lard with β-CD and changes in quality of lard after such treatment.

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MATERIALS & METHODS

Materials

Lard was obtained from Chia-Hsin company (Taichung, Taiwan) and stored at −20°C until use. β-Cyclodextrin (β-CD) was purchased from Roquette Freres Co. (France). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO). Sylon BTZ and fatty acid standard were purchased from Supelco Inc. (Bellefonte, PA). All other reagents were of reagent chemical grade from E. Merck Co. (Germany).

Cholesterol removal from a lard-water mixture

Various operating conditions for cholesterol removal from lard with β-CD were investigated. To study the effects of operating time, lard (10g) and distilled water (10g) were mixed in a beaker (150 mL), then 1, 3, 5, 7 and 10% of β-CD (based on lard weight) was added to the mixture. The mixture was stirred at 150 rpm and 27°C for 0.5, 1.0, 1.5 and 2.0 hr. To study the effects of operating temperature, lard (10g) and distilled water (10g) were mixed in a beaker (150 mL), then 1, 3, 5, 7 and 10% of β-CD (based on lard weight) was added to the mixture, respectively. The mixture was stirred at 150 rpm and 27, 40, 50 and 60°C for 1 hr. To study the effects of stirring rate, lard (10g) and distilled water (10g) were mixed in a 150 mL beaker, then 1, 3, 5, 7 and 10% of β-CD (based on lard weight) was added to the mixture, respectively. The mixture was stirred at 27°C and 100, 150 and 200 rpm for 1 hr. To study the ratio of lard to water (w/w), lard (10g) was mixed with 0, 10, 20, 40, and 80g distilled water in a 150 mL beaker, respectively. To each mixture, 1, 3, 5, 7 and 10% β-CD (based on lard weight) was added and stirred at 150 rpm and 27°C for 1 hr. For each treatment after centrifuging at 3000 rpm for 15 min, the water layer was discarded and the cholesterol in lard was determined. All treatments were run in duplicate, and analyses of all samples were run in duplicate and averaged.

Chemical analyses of lard

Chemical analyses of lard, including acid value, peroxide value, iodine value, saponification value and refractive index were performed according to AOCS (1980) methods. To determine the fatty acid content of lard, the methyl esters of fatty acids were prepared according to the AOAC (1984) procedure and analyzed by GC. The GC system was a Hitachi model G-3000 gas chromatograph equipped with a flame ionization detector (FID) and a Hitachi model D-2000 Chromato integrator. The stainless steel column (2m × 2 mm i.d.) was packed 10% SP 2330 on 100/120 Chromosorb WAW (Supelco, Bellefonte, PA). Column temperature was 210°C; injection port temperature was 240°C; the detector was set at 240°C. The carrier gas was nitrogen 30 mL/min. Fatty acids were identified via a standard mixture of methyl esters and quantified with tripentadecanoic acid as an internal standard. All determinations were performed in triplicates and mean values were reported.

Determination of cholesterol

One gram lard, to which 100 μg 5α-cholestane was added as an internal standard for gas chromatography, was saponified in 30 mL 1N KOH in methanol at 25°C for 20 hr. Saponified samples were mixed with distilled water (30 mL) and extracted three times with ethyl ether. The extracts were pooled and washed with 10 mL 0.5N KOH once and with 10 mL distilled water twice. The dried ether extracts were redissolved into 100 μL pyridine, to which 50 μL Sylon BTZ (Supelco Inc.,

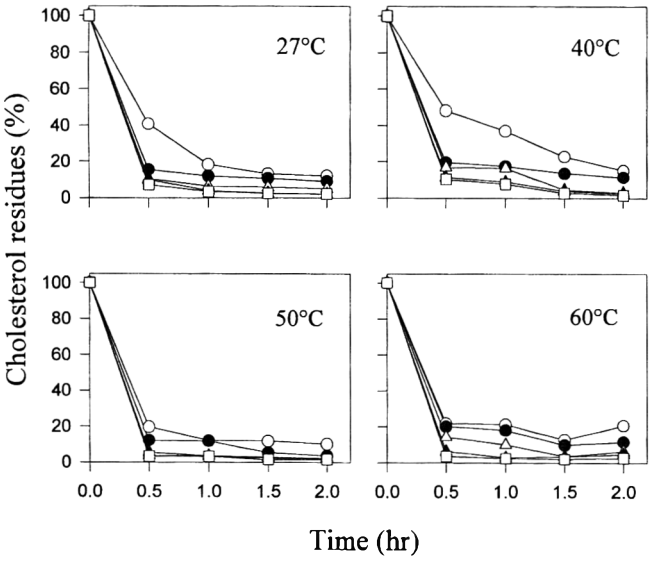

Fig. 1—Effects of stirring time and β-CD concentration on removal of cholesterol from lard by stirring at 150 rpm under different temperatures. Lard:water (1:1, w/w). - ○ -, 1% β-CD; - ● -, 3% β-CD; - △ -, 5% β-CD; - ▲ -, 7% β-CD; - □ -, 10% β-CD.

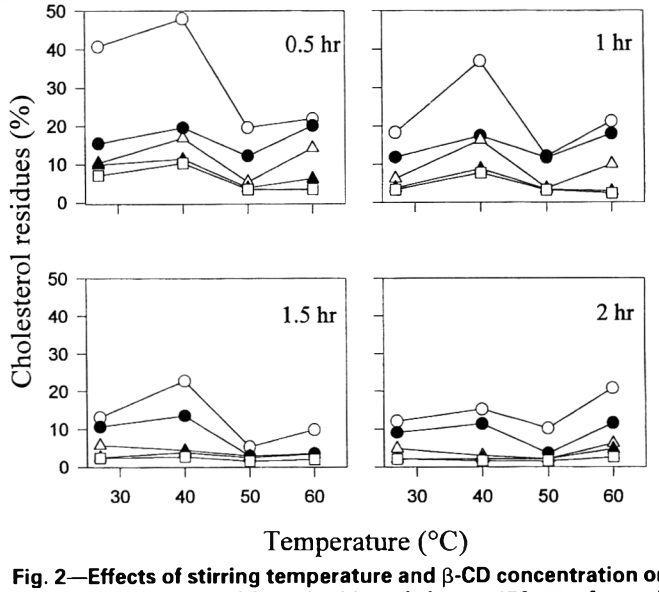

Fig. 2—Effects of stirring temperature and β-CD concentration on removal of cholesterol from lard by stirring at 150 rpm for various periods. Lard:water (1:1, w/w). - ○ -, 1% β-CD; ● -, 3% β-CD; - △ -, 5% β-CD; - ▲ -, 7% β-CD; - □ -, 10% β-CD.

Bellefonte, PA) was added to convert sterols into the corresponding trimethylsilyl (TMS) ether sterols (Park and Addis, 1985). TMS derivative of cholesterol was determined by capillary GC (Hitachi G-3000, Hitachi, Ltd., Tokyo) with a flame ionization detector using a fused silica capillary column DB-1 (15m × 0.25 mm i.d., 0.1 μm film thickness; J&W Scientific Inc., Rancho Cordova, CA) and a Hitachi D-2500 integrator. The injector was 270°C and detector 300°C. The column oven temperature was raised from 180 to 250°C at 3°C/min, and held for 5 min. Nitrogen carrier gas was used at 1.5 mL/min. Sample injection volume was 1 μL with a split ratio 1/100. Quantification of cholesterol was based on a peak area comparison and relative response factor with internal standards.

Statistical analysis

Data of acid value and peroxide value were analyzed using the Statistical Analysis System (SAS Institute Inc., 1985) software package. Significant differences between means were determined by Duncan's Multiple Range tests.

RESULTS & DISCUSSION

Chemical characteristics of lard

Properties of lard were as follows: moisture, 0.03%; refractive index (4°C), 1.458; saponification value, 202.2; iodine index, 70.9; peroxide value (meq/kg), 2.26; acid value, 0.99. The content of cholesterol in lard was 497.5 ± 12.0 ppm, with 12.9% cholesterol ester.

Effect of stirring time

The effect of stirring time on removal of cholesterol from lard by β-CD at different temperatures (Fig. 1) showed generally cholesterol removal increased with stirring time. Makoto et al. (1992) reported that 62.9, 91.1 and 94.6% cholesterol were removed from cheese by stirring with 10% β-CD at 45°C and 1:1 of water to cheese for 10, 20 and 30 min, respectively. Cholesterol removal also was slightly decreased when stirring at 60°C for 2 hr. This may be due to the instability of inclusive complex between β-CD and cholesterol at higher temperatures and longer stirring times. Oakenfull et al. (1990) indicated that 64.2, 73.1 and 63.7% cholesterol could be removed from yolk plasma by stirring with 2.7% β-CD at 40°C for 5, 10 and 15 min, respectively.

Removal of cholesterol from lard was also related to the concentration of β-CD (Fig. 1). About 90–95% cholesterol in lard was removed by stirred with 10% β-CD for 0.5 hr. According to the report of Oakenfull et al. (1990), 9.8 and 23.5% cholesterol were removed from yolk plasma by 1 and 2% β-CD, respectively.

Effect of stirring temperature

Temperature was also important for removing cholesterol from lard with β-CD. Removal of cholesterol from lard with β-CD stirred at 50°C was greater than when stirred at other temperatures. However, the efficiency was increased with increasing stirring time when treated at 40°C, but it decreased with increased stirring time when treated at 60°C (Fig. 2). Oakenfull et al. (1990) indicated that removal of cholesterol from milk with β-CD was markedly influenced by temperature. The higher removal was found at a lower temperature, i.e., 77, 63 and 62% cholesterol in milk were removed when treated with β-CD at 4, 8 and 40°C, respectively.

Effect of stirring rate

The removal was increased with an increasing stirring rate (Fig. 3). In addition, 90% cholesterol in lard was removed with 7% β-CD when stirred at 150 or 200 rpm for 1 hr.

Effect of ratio of lard to water

Removal of cholesterol from lard by β-CD was markedly related to the ratio of lard to water (Fig. 4). The highest efficiency for removal of cholesterol from lard by β-CD occurred at 1:1 ratio of lard to water. At that condition, 90% cholesterol in lard was removed by stirring with 5% β-CD for 1 hr. The lower removal of cholesterol occurred for the lower ratio of lard to water. This may be due to the fact that higher water content would reduce the inclusion between β-CD and cholesterol. Pierrick and Leon (1981) reported that 25% cholesterol in cheese was removed by stirring with 5% β-CD at 40°C and at 1:1 ratio of cheese to water for 5 hr. A small amount of cholesterol could be removed by β-CD without addition of water (Fig. 4). Since the outer shell of β-CD has hydrophilic properties, the inner hydrophobic layer of β-CD would become exposed when directly interacting with oil, as well as reducing its inclusion capability. Therefore, the proper addition of water during operation is necessary. According to the result of Courrege-

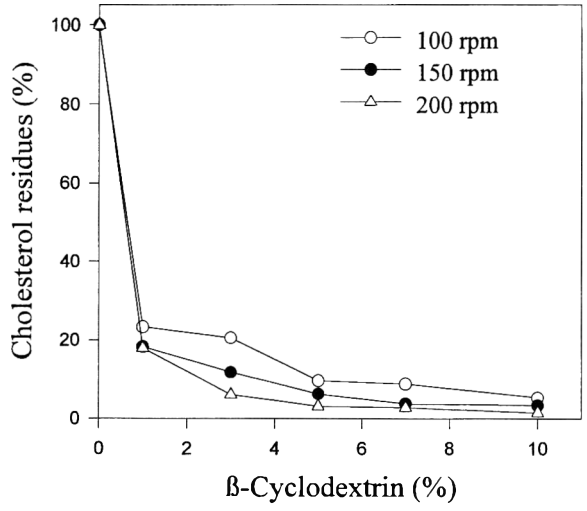

Fig. 3—Effects of stirring rate and β-CD concentration on removal of cholesterol from lard at 27°C for 1 hr. Lard:water (1:1, w/w). - ○ -, 100 rpm; - ● -, 150 rpm; - △ -, 200 rpm.

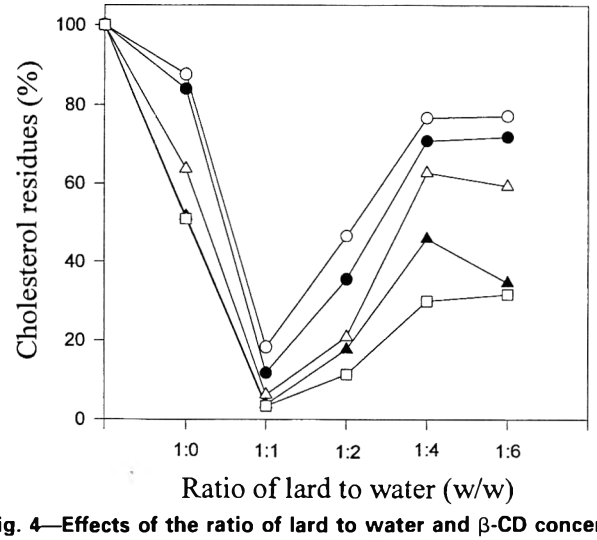

Fig. 4—Effects of the ratio of lard to water and β-CD concentration on removal of cholesterol from lard by stirring at 27°C and 150 rpm for 1 hr. - ○ -, 1% β-CD; - ● -, 3% β-CD; - △ -, 5% β-CD; - ▲ -, 7% β-CD; - □ -, 10% β-CD.

longue et al. (1989), 26 and 33% cholesterol were removed from dehydrated butter by 5 and 10% β-CD, respectively, without addition of water. In addition, 17 and 18% cholesterol in suet and lard were removed by 5% β-CD. However, all of the β-CD inclusive complexes after treatments were removed from the sample by mixing with water and centrifugation.

Change in acid value of β-CD treated lard

The acid value in β-CD treated lard decreased with increasing stirring period and β-CD concentration ($P < 0.05$) (Fig. 5). The acid value in β-CD treated lard was generally lower than that of untreated lard (AV 0.99). This might be due to the fact that the free fatty acid in lard was also removed by β-CD during removing cholesterol. The acid value of lard showed an increase when treated with a low level of β-CD (1 and 3%) for 0.5 hr, then decreased with longer reaction times. The acid value of lard was increased when treated with β-CD at 27°C for 1.5 hr. This might have been caused by the fact that the inclusion of β-CD with cholesterol was greater than with free fatty acid, since the cholesterol in lard was effectively removed at that temperature (Fig. 1). Roderbourg et al. (1990) indicated that 65% cholesterol and 38% free fatty acid in milk fat were reduced after being treated with β-CD.

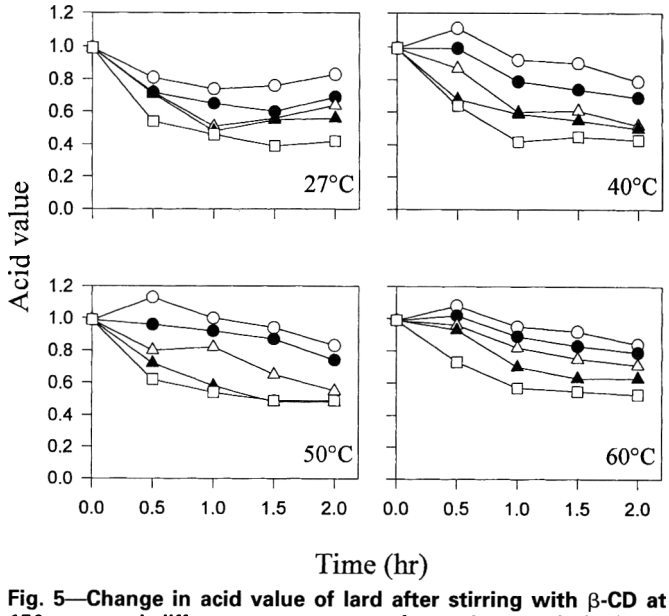

Fig. 5—Change in acid value of lard after stirring with β-CD at 150 rpm and different temperatures for various periods. Lard: water (1:1, w/w). - ○ -, 1% β-CD; - ● -, 3% β-CD; - △ -, 5% β-CD; - ▲ -, 7% β-CD; - □ -, 10% β-CD.

In general, although the acid value of lard increased with increasing treating temperatures, the acid value of β-CD treated lard still decreased with increasing concentration of β-CD (Fig. 6). Thus the change in acid value of β-CD treated lard was related to the treating temperature and to the β-CD concentration.

The acid value of β-CD treated lard remained unaffected by stirring rate (Fig. 7). The acid value of β-CD treated lard with 150 rpm stirring rate was lower than the other two stirring rates (100 or 200 rpm), but it was not different ($P > 0.05$) between 150 rpm and 100 or 200 rpm at 5 and 7% β-CD. The acid value in lard also decreased with increasing β-CD concentration.

The acid value of β-CD treated lard apparently was not ($P > 0.05$) influenced by the ratio of lard to water (data not shown). The lower acid value was found in β-CD treated lard with 1:1 ratio of lard to water. The acid value of β-CD treated lard also did not decrease at any β-CD concentration without addition of water. This may indicate that the structure of β-CD molecule was changed in the absence of water (Makoto et al., 1992).

Change in peroxide value of β-CD treated lard

The effects of stirring time (Table 1) and stirring temperature (Table 2) on peroxide value of β-CD treated lard indicated that the peroxide of β-CD treated lard increased ($P < 0.05$) when compared with untreated lard. However, the increased peroxide value of β-CD treated lard remained uninfluenced by stirring time, stirring temperature, or β-CD concentration.

The peroxide value of β-CD treated lard increased with increasing stirring rate (Table 3). The increasing peroxide value in β-CD treated lard with a higher stirring rate might be due to dissolving more air at a high stirring rate.

Peroxide value in β-CD treated lard was markedly increased with addition of water (Table 4), while the POV of β-CD treated lard increased ($P < 0.05$) when water was added. In addition, the fatty acid composition of lard apparently was not changed after being treated with β-CD at any testing conditions (data not shown).

CONCLUSION

REMOVAL OF CHOLESTEROL from a lard-water mixture increased with increasing β-CD concentration. About 90% of cholesterol could be removed from lard by 5% β-CD with 1:1 ratio of lard

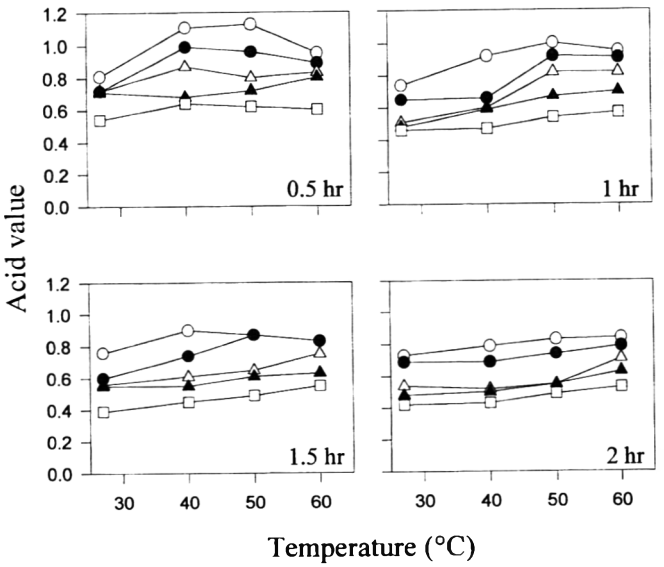

Fig. 6—Effects of stirring time and β-CD concentration on acid value of lard by stirring at 150 rpm under different temperatures. Lard:water (1:1, w/w). - ○ -, 1% β-CD; - ● -, 3% β-CD; - △ -, 5% β-CD; - ▲ -, 7% β-CD; - □ -, 10% β-CD.

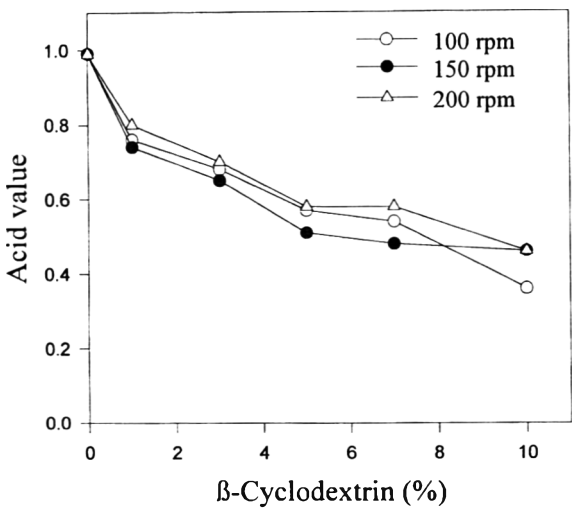

Fig. 7—Change in acid value of lard after stirring with β-CD at 27°C and various stirring rates for 1 hr. Lard:water (1:1, w/w).

Table 1—Effects of stirring time and β-CD concentration on the peroxide value of lard

Stirring[a] time (hr)	Peroxide value (meq/kg)				
	1% β-CD[b]	3% β-CD	5% β-CD	7% β-CD	10% β-CD
Control[c]	2.26a[d]	2.26a	2.26a	2.26a	2.26a
0.5	4.28b	4.64bc	4.58bc	4.71bc	4.54bc
1	4.32b	5.00c	4.77bc	4.48bc	4.61bc
1.5	4.24b	4.32b	4.54bc	4.50bc	4.52bc
2	4.51bc	4.81c	4.32b	4.41b	4.28b

[a] Stirring temp, 27°C; stirring rate, 150rpm; lard:water = 1:1 (w/w).
[b] The percentage of β-CD relative to lard (w/w).
[c] Lard was not through treatment.
[d] The mean values in each cclumn with the same letters are not significantly different at 5% level by Duncan's multiple range test.

to water, stirring at 150 rpm and 27°C. The addition of water is important to increase efficiency; however, too much water would reduce the interaction between β-CD and cholesterol. Free fatty acids in lard were also removed by β-CD; however, the fatty acid composition of lard did not change after being treated with β-CD. Therefore, β-CD is suitable for removing cholesterol from lard and retain its quality.

Table 2—Effects of stirring temperature and β-CD concentration on the peroxide value of lard

Stirring[a] temp (°C)	Peroxide value (meq/kg)				
	1% β-CD[b]	3% β-CD	5% β-CD	7% β-CD	10% β-CD
Control[c]	2.26a[d]	2.26a	2.26a	2.26a	2.26a
27	4.32b	5.00c	4.77c	4.48bc	4.61bc
40	4.75c	4.10b	4.35b	4.52bc	4.62bc
50	4.77c	4.70c	4.39b	4.94c	4.16b
60	4.86c	4.25b	4.11b	4.93c	4.48bc

[a] Stirring rate, 150 rpm; stirring time, 1 hr; lard:water = 1:1 (w/w).
[b] The percentage of β-CD relative to lard (w/w).
[c] Lard was not through treatment.
[d] The mean values in each column with the same letters are not significantly different at 5% level by Duncan's multiple range test.

Table 3—Effects of stirring rate and β-CD concentration on the peroxide value of lard

Stirring[a] rate (rpm)	Peroxide value (meq/kg)				
	1% β-CD[b]	3% β-CD	5% β-CD	7% β-CD	10% β-CD
Control[c]	2.26a[d]	2.26a	2.26a	2.26a	2.26a
100	3.24b	3.43b	3.40b	3.24b	3.21b
150	4.32c	5.00c	4.77c	4.48c	4.61c
200	5.18d	5.58d	5.01c	4.98d	5.30d

[a] Stirring temp, 27°C; stirring time, 1 hr; lard:water = 1:1 (w/w).
[b] The percentage of β-CD relative to lard (w/w).
[c] Lard was not through treatment.
[d] The mean values in each column with the same letter are not significantly different at 5% level by Duncan's multiple range test.

Table 4—Effects of the ratio of lard to water and β-CD concentration on the peroxide value of lard

Ratio of[a] lard:water (w/w)	Peroxide value (meq/kg)				
	1% β-CD[b]	3% β-CD	5% β-CD	7% β-CD	10% β-CD
Control[c]	2.26a[d]	2.26a	2.26a	2.26a	2.26a
1:0	2.49b	2.84b	2.42b	2.84b	2.87b
1:1	4.32c	5.00d	4.77c	4.48d	4.61c
1:2	4.51d	4.92d	4.64bc	4.37c	4.70cd
1:4	4.35c	4.81c	4.94d	4.93e	4.73d
1:6	4.85e	4.74c	4.91d	4.92e	4.73d

[a] Stirring temp, 27°C; stirring rate, 150 rpm; Stirring time, 1 hr.
[b] The percentage of β-CD relative to lard (w/w).
[c] Lard was not through treatment.
[d] The mean values in each column with the same letters are not significantly different at 5% level by Duncan's multiple range test.

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Postmortem Delay Time and Heating Rate Affect Tenderness and Ultrastructure of Prerigor Cooked Bovine Muscle

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ABSTRACT

The response of prerigor bovine muscle to different heating conditions was determined. Samples were fast-heated at six postmortem times or at five heating rates at four early postmortem times. All samples were heated to 70°C internally. Prerigor muscle cooked at progressively shorter postmortem times and faster heating rates had correspondingly lower shear values. In prerigor fast-heated samples, phase contrast microscopy revealed transverse bright stripes along the myofiber while scanning electron microscopy showed transverse surface wrinkles, bulges, interior contraction bands, and stretched areas. Postmortem time and heating should be controlled to improve beef tenderness.

Key Words: bovine muscle, heating rate, tenderness, SEM micrographs

INTRODUCTION

THE HOT-BONING PROCESS has many advantages over cold-boning, e.g., reduced refrigeration energy, cooler space, and in-plant holding time (Reagan, 1983). Prerigor cooking would take advantage of many of the benefits of hot-boned meat. Although improved tenderness is known to result from prerigor cooked meat, this practice has not been employed commercially. This is probably because of inconsistent palatability of prerigor cooked meat.

Marsh (1964) found neck muscle (ox sternomandibularis) more tender cooked in boiling water within 1–2 hr postmortem than that cooked after 24 hr. Cia and Marsh (1976) found that prerigor cooked beef muscle (sternomandibularis) was more tender than postrigor cooked muscle if cooked rapidly within about 3 hr of slaughter. May et al. (1962) cooked boneless pork chops from 4 muscles (longissimus dorsi, semitendinosus, semimembranosus, and biceps femoris) and found that tenderness decreased with onset of rigor mortis in the first 7 hr postmortem. Dransfield and Rhodes (1975) found that rapid heating soon after slaughter produced similar shear values as postrigor samples for prerigor beef sternomandibularis.

Other researchers have reported prerigor cooked meat to be less tender than postrigor cooked meat. Henning et al. (1973) cooked samples from three bovine muscles (biceps femoris, semitendinosus, and semimembranosus) at 3 postmortem times (1 hr, 48 hr, and 5 days) and found tenderness increased from the first sampling time to the third. Ray et al. (1983) cooked beef semimembranosus and semitendinosus roasts and Loucks et al. (1984) cooked beef semimembranosus roasts at 1 hr and 48 hr postmortem and found that shear values and sensory panel ratings showed that prerigor cooked roasts were less tender than postrigor cooked roasts. Shin et al. (1993) cooked pork and beef roasts from triceps brachii in a conventional oven at 150, 200, or 250°C and reported that prerigor cooked roasts were less tender than postrigor cooked roasts.

The inconsistent tenderness of prerigor cooked meat may result from employing different postmortem times, heating rates, species, and/or muscles. For example, Paul et al. (1952) used deep fat frying to rapidly heat steaks while heating roasts much more slowly in a conventional oven. They found the roasts to be least tender soon after slaughter but increasingly more tender as postmortem time lengthened. However, steaks were most tender immediately after slaughter and became less tender up to 24 hr postmortem.

Ultrastructural changes may help explain changes in tenderness of prerigor cooked meats. Weidemann et al. (1967) used optical light microscopy (OLM) and transmission electron microscopy (TEM) to examine the ultrastructure of prerigor and postrigor cooked ox semitendinosus muscle and found supercontraction clots on both types of micrographs. Hsieh et al. (1980) using TEM and scanning electron microscopy (SEM) to examine the ultrastructure of prerigor cooked bovine sternomandibularis found supercontraction bands alternating with fragmented areas on TEM micrographs. Their SEM micrographs showed a wavy, wrinkled surface appearance on fibers from prerigor cooked muscle. These researchers (Weidemann et al., 1967; Hsieh et al., 1980) have demonstrated relationships between meat ultrastructure and shear force. However, ultrastructural changes in prerigor muscle upon different heating conditions have not been reported.

Our objective was to determine the influence of postmortem delay time and heating rate on the tenderness of prerigor cooked meat and consider ultrastructural differences that may cause changes in tenderness for prerigor cooked meat using OLM and SEM.

MATERIALS & METHODS

THIS STUDY consisted of three experiments. The preliminary experiment investigated the effects of postmortem delay time on tenderness of prerigor cooked beef to determine appropriate delay times for subsequent studies. In the second experiment, effects of heating rate and postmortem delay time on tenderness and ultrastructural changes of prerigor cooked beef were measured. Phase contrast light microscopy (an OLM) was used for ultrastructural examination. In the third experiment, ultrastructural changes associated with prerigor cooking of bovine muscle were examined using SEM.

Effects of postmortem delay time on tenderness

One bovine sternomandibularis muscle from each of two steers was obtained immediately after exsanguination. Each muscle was cut into 3 cross-sections. Since all cutting was done at very early postmortem times (within 20–30 min), muscle relaxed after the contraction due to cutting. Thus no additional protection was required from muscle shortening and we could maintain samples in relatively relaxed state upon cooking.

Six postmortem delay times before cooking were studied (0.5, 3, 6, 9, 12, and 24 hr). Samples from steer 1 were given delay times of 3, 9 and 24 hr and those from steer 2 were given delay times of 0.5, 6 and 12 hr. Postmortem delay time was nested within steers. Samples were further divided into halves and kept in Ringer's solution (0.86% NaCl, 0.033% $CaCl_2$ and 0.03% KCl) at about 20°C until cooking except 0.5 hr delay time samples which were cooked immediately.

Shear force. The samples were cooked to internal temperature 70°C in an oil bath at 90°C and monitored with a piercing digital Heat-Prober™ thermometer (William Hahl Co., Los Angeles, CA). Average heating rate calculated after the experiment was 4.45 ± 0.75°C/min. Cooked muscle samples were placed individually in 3 mil polyethylene bags and cooled overnight at 3°C. They were held at about 20°C for 30 min and then cut into 10 × 10 mm rectangular cores with long axis parallel to

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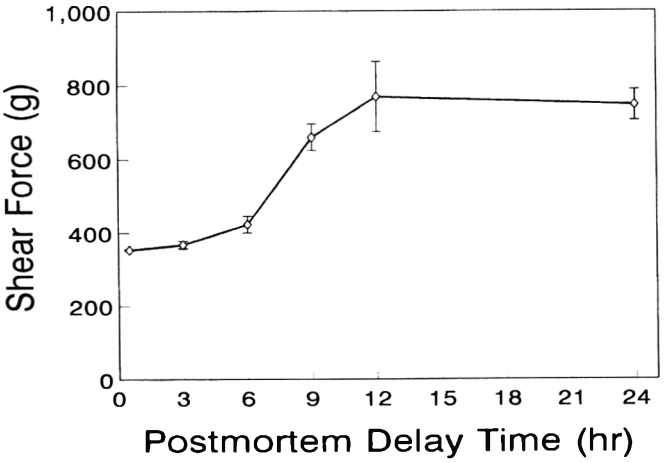

Fig. 1—Shear values of bovine sternomandibularis muscles heated rapidly (4.45 ± 0.75°C/min) in early postmortem (0.5---24 hr).

the fibers. Sample cores (2–4/muscle sample) were sheared perpendicular to fiber using a Warner-Bratzler shear (model SD-50, John Chatillon & Sons, New York, NY). Maximal forces were recorded as shear values.

Effects of heating rate and postmortem delay time

Sternomandibularis muscles from eight steers were assigned to four postmortem delay times of < 6 hr (0.5, 1, 3 and 6 hr) before cooking. The further heating rate investigation was conducted within the first 6 hr postmortem, because the preliminary study showed that most tenderness improvement was achieved within the first 6 hr postmortem. Muscles were kept in Ringer's solution at about 20°C and excised into six pieces prior to cooking. One sample piece from each muscle was randomly selected for determination of muscle pH before cooking. The other five samples were randomly assigned to a target heating rate (4.5, 1.5, 0.8, 0.5 or 0.4°C/min). Two additional sample pieces from one of the steers were used as postmortem reference samples and cooked either fast (4.5°C/min) or slow (0.5°C/min) at 48 hr postmortem. This experiment was analyzed as a two-factor design (delay time and heating rate) with repeated measures on heating rate. Delay time was completely randomized across muscles. For heating rate, the experiment was a randomized block design with muscle as the block (Neter et al., 1985).

Cooking procedure. Each target heating rate (4.5, 1.5, 0.8, 0.5, and 0.4°C/min) was achieved by a heating system which consisted of a beaker with cold or preheated oil and a heating plate pre-adjusted to heat the sample from ambient (about 20°C) to 70°C in heating times of 10, 30, 60, 90 or 120 min. Internal temperature was monitored using thermocouples (type K) and a portable data logger (RM 1242, Cole Parmer Instrument Co., Chicago, IL). Sample weights were measured before and after cooking and difference was recorded as cooking loss. Cooked samples were cooled and sheared. Maximal forces were recorded as shear values.

Muscle pH. The pH values were measured using a digital pH meter (Orion model 701, Cambridge, MA) before and after cooking by homogenizing 5g sample in 45 mL of a solution containing 5 mM sodium iodoacetate and 150 mM potassium chloride (Solomon, 1987).

Samples for phase contrast microscopy. Cooked meat (≈1g) from the interior of samples was homogenized for 30 sec using a mini-sample blender (Eberbach corporation, Ann Arbor, Mich) with a Waring Blendor base (Waring Products division of Dynamics corporation of American, Winsted, Conn) in about 24 mL distilled water. One drop of homogenate with visible fibers was examined on the slide using phase contrast light microscopy (Zeiss Axioskop) without further preparation. Photographs were taken with ISO 100 and TMAX films.

Ultrastructural changes related to prerigor cooking

Bovine sternomandibularis muscles from one steer were taken 15 min after exsanguination. One muscle was used prerigor and subjected to heat treatment at 0.5 hr postmortem. The other (postrigor) muscle was held for 24 hr at 16°C to ensure complete rigor mortis and minimal shortening (Locker and Hagyard, 1963), then held for another 24 hr at 3°C for a total of 48 hr before being heated.

Before heating treatment, muscles were divided into three samples each by cross-cutting. One sample served as unheated control. The other two had thermocouples inserted and were heated in oil baths at a target heating rate of 4.5°C/min (fast-heating) or 0.5°C/min (slow-heating) from ambient temperature (about 35°C for prerigor samples and 5°C for postrigor samples) to an internal temperature of 70°C.

Samples for SEM. SEM was performed at the Electron Microscope Facility for the Life Sciences in the Biotechnology Institute at Penn State University. Raw and cooked muscle samples were pre-fixed with 3% glutaraldehyde in 0.15M sodium cacodylate buffer at pH 7.1 overnight at 3°C. All pre-fixed samples were washed three times with 0.15M sodium cacodylate buffer for 3 min each, then post-fixed with 1% osmium tetroxide in 0.15 M sodium cacodylate buffer (pH 7.1) at about 20°C for 1 hr.

The post-fixed samples were washed 3 times with double distilled water for 3 min each and dehydrated by gradient ethanol series (10%, 25, 40, 50, 70, 85, 95, 100, 100, and 100%) for 7 min each. Samples remained covered with alcohol throughout the ethanol-drying process.

The ethanol-dried samples were then frozen in liquid nitrogen and cryofractured by a razor on a copper block immersed in liquid nitrogen. All tools contacting samples were cooled in liquid nitrogen before contact. Cryofractured samples were thawed in absolute alcohol, transferred into critical-point-drying (CPD) carriers, which were also immersed in absolute alcohol, and capped. Samples in CPD carriers were critical-point-dried in a Fisons Polaron E3000 (Energy Beam Sci. Inc., Agawan, MA) with four exchanges of liquid carbon dioxide.

The critical-point-dried samples were mounted on aluminum stubs. The mounting adhesive and exposed aluminum stub were coated with a small amount of colloidal silver to prevent electron accumulation when examined under SEM. The mounted samples were sputter-coated with gold to a thickness of about 42 nm before examination under a scanning electron microscope (Topcom Inc., model 60, Pleasanton, CA).

Statistical analysis

Shear force values were analyzed by analysis of variance using Statistical Analysis System (SAS Institute, Inc., 1985). Means were calculated by Least Square Means procedure. The preliminary study was a one-way analysis and the main study was a split plot design. A significant interaction was found between postmortem time and heating rate in the main study. Differences between consecutive points in the preliminary study and the effect of one factor when the other was fixed in the main study were tested by Bonferroni t-test (Neter et al., 1985).

RESULTS & DISCUSSION

Delay time effect (preliminary study)

Prerigor bovine sternomandibularis muscle samples were cooked with a fast heating rate at 6 different postmortem times (0.5, 3, 6, 9, 12 and 24 hr) in the preliminary study. The shear values of the cooked muscle samples were compared (Fig. 1). The mean shear values increased with delay time from 0.5 hr postmortem up to 12 hr with the greatest increase ($p < 0.01$) between 6 and 9 hr postmortem. Other changes between two consecutive points were not significant ($p > 0.01$). The predicted regression line for the first 12 hr postmortem was

$$Y = 339 + 4X + 3X^2$$

where Y is the predicted shear force value (g/cm²) and X is the postmortem delay time (hr). This model resulted in an $R^2 = 0.92$. Thus, postmortem delay time explained 92% variation of shear force values.

This result confirmed the finding of Marsh (1964) that bovine neck muscles were remarkably tender if cooked within 1–2 hr postmortem and toughness increased rapidly with delay between slaughter and cooking. Cia and Marsh (1976) also reported that bovine sternomandibularis was more tender if cooked within about 3 hr of slaughter compared to that in which rigor was established.

Some investigators (Cia and Marsh, 1976; Khan, 1974; Abugroun et al., 1985a,b) reported that muscle shortening for shorter delay time samples was more severe than for those cooked after a longer delay time. Structural damage resulting from severe

shortening during prerigor cooking might account for reduction in shear force observed in muscle heated up to 6 hr postmortem in our study. As rigor progressed, the responsiveness of the muscle declined so that samples heated at 12 hr postmortem showed no improvement in tenderness.

Delay time effect as related to heating rate

Muscles were randomly assigned to one of four postmortem times. Samples from each muscle were then cooked at one of 5 heating rates. Shear force of cooked samples was compared after cooling overnight (Fig. 2). For muscle samples subjected to faster heating rates (4.5 and 1.5°C/min), shorter delay time resulted in reduced shear force ($p < 0.006$). At slower heating rates (0.8, 0.5 and 0.4°C/min), shorter delay time did not affect shear force.

Muscle pH and cook losses

Selected pH values and cook losses during heating for prerigor cooked beef samples were compared (Table 1). The sample pH before cooking did not change ($p > 0.05$) with postmortem delay time. This may have been due to the fairly large variation in pH value at death and inherent limitations in prerigor pH measurements. The pH of cooked prerigor muscle samples correlated with heating rate ($p < 0.01$). The prediction equation was:

$$Y = 6.27 + 0.29X - 0.05X^2 \qquad R^2 = 0.71$$

where Y is the predicted pH value and the X is the heating rate. A plot (not shown) of pH values of cooked prerigor samples to heating rate indicated that in early postmortem 1°C/min in heating rate could be critical in prerigor cooking for obtaining relatively high cooked muscle pH values. Cook loss correlated with the reciprocal of heating rate ($p < 0.05$) in the following fashion:

$$Y = 22.34 - 26.73X + 24.44X^2 - 6.11X^3 \qquad R^2 = 0.44$$

where Y is the predicted cook loss (%) and X is the reciprocal of heating rate. A plot (not shown) of cook loss to heating rate indicated that high cooking losses might be associated with high heating rates and also with relatively slow heating rates of 0.5 to 0.7°C/min.

Heating rate effect

Shear force values (Fig. 2) represent three observations each for 0.5 hr and 6 hr postmortem samples, two observations each for 1 hr and 3 hr postmortem samples and 1 observation for 48 hr reference samples. Shear values for prerigor samples cooked at postmortem times up to 6 hr decreased with increasing heating rate ($p < 0.006$). Prerigor cooked samples had lower shear values than postrigor cooked reference samples.

Following are the predicted regression equations of shear value for each delay time:

0.5 hr	$Y = 878.1 - 351.4X + 54.1X^2$	$R^2 = 0.71$
1 hr	$Y = 837.1 - 78.1X$	$R^2 = 0.87$
3 hr	$Y = 913.9 - 102.7X$	$R^2 = 0.83$
6 hr	$Y = 874.8 - 154.3X + 19.6X^2$	$R^2 = 0.93$

where Y is the predicted shear force value (g/cm²) and X is the heating rate (°C/min).

Our results generally confirmed related reports. For example, Shin et al. (1993) using a heating rate of <1°C/min reported no improvement in tenderness. Our results showed tenderness increased with increasing heating rate and supported the finding of Paul et al. (1952) that slow heating of prerigor cooked beef toughened roasts while very rapid heating of thinner steaks in deep fat frying tenderized them. Abugroun et al. (1985a) also reported that rapid heating (2°C/2 min) produced more tender prerigor cooked products than did prerigor slow heating (2°C/12 min) for bovine triceps brachii muscle. Some investigators (Cia and Marsh, 1976; Khan, 1974; Abugroun et al., 1985a,b; Silva et al., 1993) also reported that muscle shortening for faster heating rate samples was more severe than for those cooked at slower heating rates.

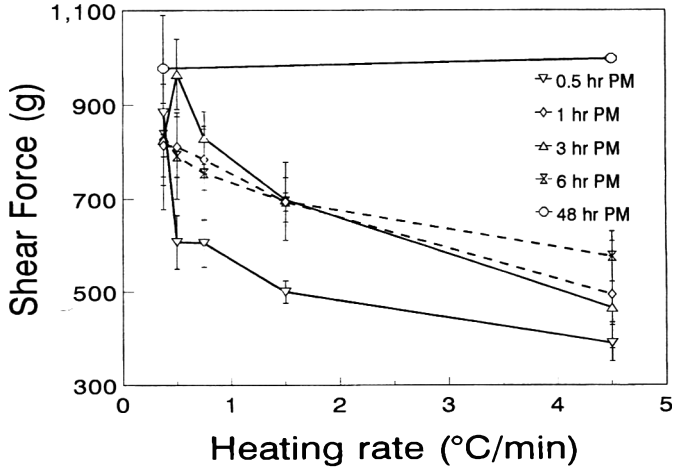

Fig. 2—Shear values of bovine sternomandibularis muscles cooked as related to postmortem times and heating rates. PM, postmortem.

Table 1—pH value and cooking loss of prerigor cooked beef

Delay time (hr)	Actual heating rate (°C/mir)	pH Before cooking	pH After cooking	Cooking loss (%)
0.5	3.1	—[a]	6.21	14.59
	1.3		6.35	13.12
	0.9		6.35	11.74
	0.7		6.37	15.39
	0.4		6.25	8.41
0.5	1.5	6.96	6.59	16.86
	1.2		6.54	16.44
	0.9		6.51	16.56
	0.5		6.45	17.93
	0.4		6.27	13.61
1.0	4.2	6.82	6.68	
	1.3		6.59	
	0.9		6.51	
	0.7		6.45	
	0.5		6.45	
6.0	4.2	6.50	6.67	17.79
	1.3		6.57	12.42
	0.9		6.53	14.45
	0.5		6.48	18.89
	0.4		6.40	12.57
6.0	4.5	6.77	6.62	17.25
	1.4		6.53	13.88
	1.1		6.42	10.12
	0.7		6.54	16.16
	0.4		6.22	15.99
48.0	3.8	5.68	6.00	
	0.6		5.90	

[a] Not measured.

Phase contrast light microscopy

Although samples were examined with no further preparation, the striation of muscle fibers was easily visible. Of >100 light micrographs, only photos showing the most common features are included here. Examination of fiber fragments prepared from prerigor, fast-heated muscle revealed a distinctive myofibrillar banding pattern (Fig. 3) not seen in any postrigor cooked muscles we examined. The postrigor skeletal muscle fibers showed

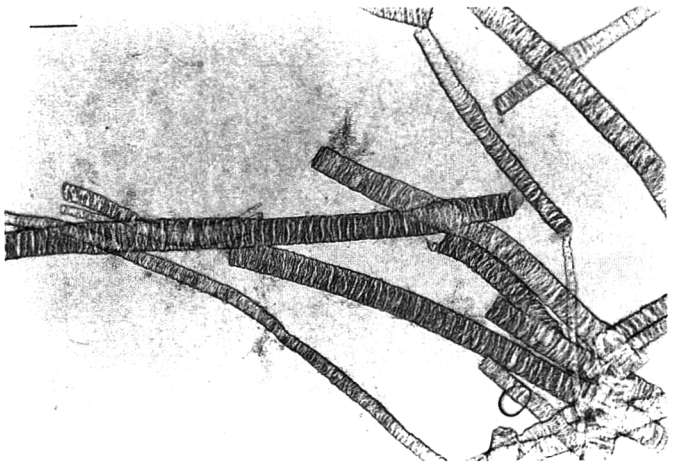

Fig. 3—Phase contrast micrograph of prerigor (0.5 hr postmortem) fast-heated (about 4.5°C/min) bovine sternomandibularis muscle fibers showing bright and dark irregular transverse stripes. Bar = 100μ.

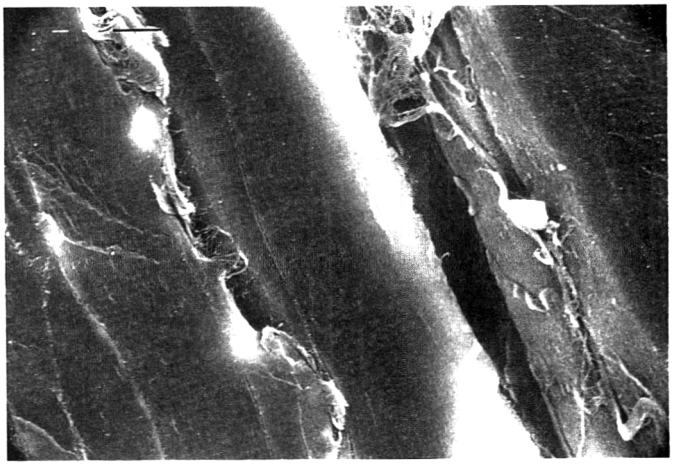

Fig. 5—SEM micrograph of prerigor (0.5 hr postmortem) uncooked muscle sample showing cryofractured fiber interior. Final bar = 10μ.

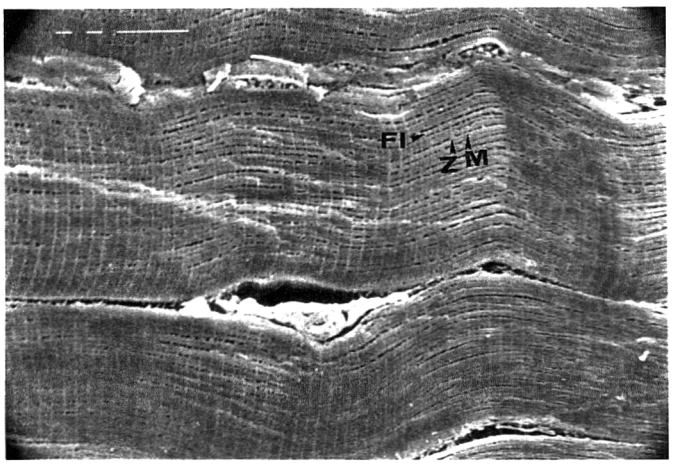

Fig. 4—SEM micrograph of postrigor (48 hr postmortem) uncooked muscle sample showing myofibrils (FI), Z lines (Z), and M lines (M). Final bar = 10μ.

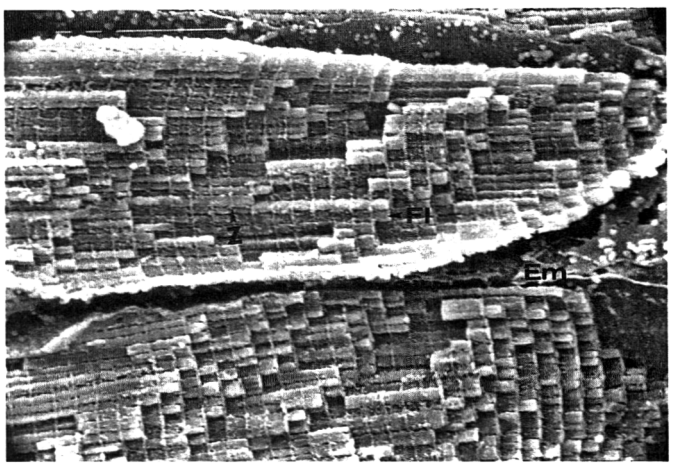

Fig. 6—SEM micrograph of postrigor (48 hr postmortem) slow-heated (0.7°C/min) muscle sample showing myofibrils (FI), Z disks (Z), endomysium (Em), and breakage along myofibrils. Final bar = 10μ.

regular transverse banding patterns along the fiber. However, in the prerigor fast-heated muscle fibers, intense irregular bands, running transversely to the long axis of the fiber appeared. These were similar to the supercontraction clots reported by Weidemann et al. (1967) in prerigor cooked ox semitendinosus muscle and to the dense banding in porcine muscles after extremely rapid postmortem anaerobic glycolysis (Cassens et al., 1963a).

Scanning electron microscopy

The microstructural changes of prerigor fast- and/or slow-heated samples were compared with controls (prerigor uncooked, postrigor uncooked, and postrigor cooked with fast or slow heating rate) using SEM. Several distinct microstructures were identified in prerigor cooked samples. All SEM micrographs were longitudinal cryofractured interior unless otherwise specified. Of about 100 SEM micrographs a few with most common features are included here.

In postrigor (48 hr postmortem) uncooked control samples (Fig. 4), Z lines and M lines were visible in the cryofractured myofiber interior. Myofibrils were partially loosened from each other. However, many remained unseparated. The sarcomere length here was 1.71μ, which was less than the 2.0–2.5μ expected for relaxed muscles (Voyle, 1969; Marsh and Carse, 1974). However, Jones et al. (1976) demonstrated that each step in SEM sample preparation contributed some degree of shortening in sarcomere length up to a total of 24%. Such shortening might account for the unusually short sarcomeres we found.

In the cryofractured interior of prerigor (0.5 hr postmortem) uncooked control fibers (Fig. 5) the fiber at the far right shows cryofractured interior as well as fiber surface with some connective tissue. Myofibrils are closely packed within the fibers. No separation of myofibrils or obvious Z disks were observed as in postrigor samples (Fig. 4, 6 and 7). The cryofractured interior surface thus looks "smooth" when compared to those of postrigor samples. No obvious banding patterns could be seen in the cryofractured myofiber interior. Transverse and longitudinal structures were barely apparent. Hence, no precise sarcomere length could be determined.

In postrigor (48 hr postmortem) slow-heated (0.7°C/min) control samples (Fig. 6), myofibrils and Z disks were apparent, two muscle fibers are shown. Shrinkage of myofibrils during cooking and/or SEM sample preparation may allow Z disks to protrude and thus become more visible. All breakage of myofibrils was at Z disks. This indicated that the area of Z disks might be the weakest point along the myofibrils for postrigor samples. This confirmed results of many other researchers (Cheng and Parrish, 1976; Jones et al., 1977; Hearne et al., 1978; Davey and Dickson, 1970; Leander et al., 1980). The sarcomere length from this micrograph was 1.72μ.

In postrigor (48 hr postmortem) fast-heated (4.5°C/min) samples, myofibrillar shrinkage was obvious (Fig. 7). The Z disks

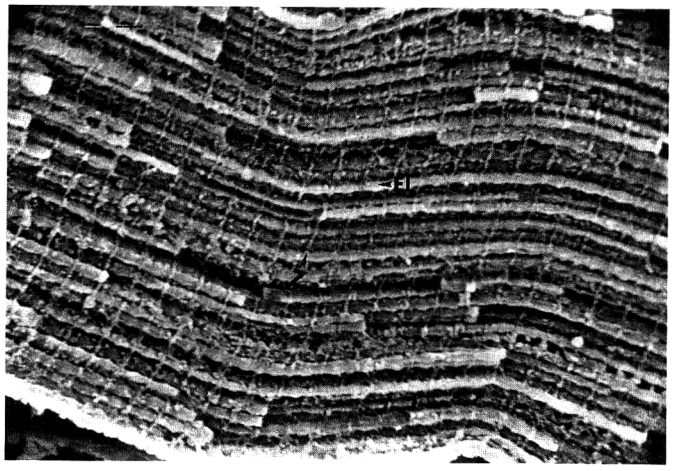

Fig. 7—SEM micrograph of post-rigor (48 hr postmortem) fast-heated (4.5°C/min) muscle fiber showing myofibrils (FI), Z lines (Z), and breakage along the myofibrils. Final bar = 1μ.

Fig. 9—SEM micrograph of prerigor (0.5 hr postmortem) fast-heated (3.9°C/min) muscle sample showing surface wrinkles (W), surface bulges (B), interior contraction bands (C), and interior stretched areas (S). Final bar = 10μ.

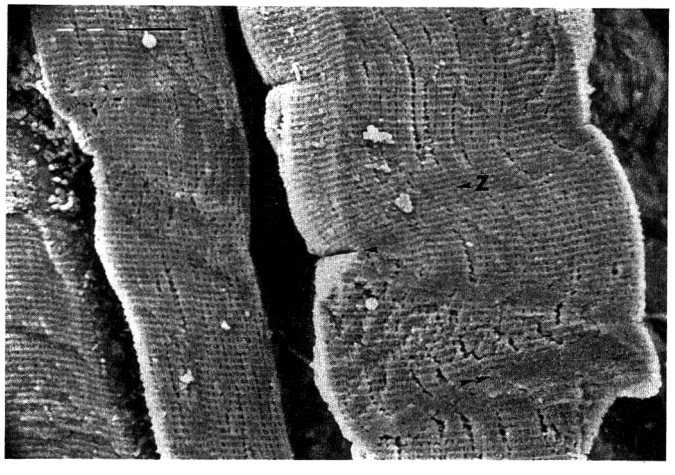

Fig. 8—SEM micrograph of prerigor (0.5 hr postmortem) slow-heated (0.5°C/min) muscle sample showing clefts (arrow) on fiber surface and Z lines (Z) in interior dark shadows (double arrows). Final bar = 10μ.

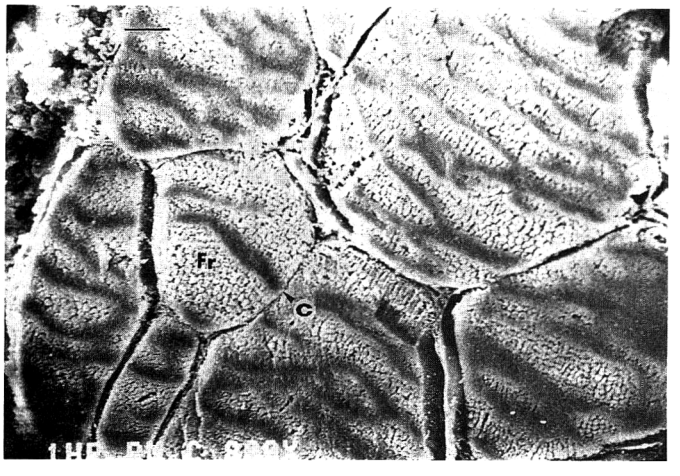

Fig. 10—SEM micrograph of prerigor (1 hr postmortem) fast-heated (about 4.5°C/min) muscle sample showing contraction bands (C) on cross-sectional cryofractured fiber (Fr) interior. Final bar = 10μ.

and myofibrils were clearly visible in the cryofractured fiber interior. As in slow-heated samples, myofibrils seemed to break adjacent to Z disks. All these features resembled those of postrigor slow-heated samples (Fig. 6). The sarcomere length from this micrograph was 1.59 μ. The similarity of features between postrigor fast- or slow-heated samples supported the finding that postrigor reference samples (slow- and fast-heated) had similar shear forces.

In prerigor (0.5 hr postmortem) slow-heated (0.5°C/min) samples, myofibrils and Z lines were obvious (Fig. 8). Clefts on fiber surfaces (indicated by an arrow) are apparent. Some dark shadows in cryofractured fiber interiors (indicated by double arrows) were present but much less obvious than clefts. These dark shadows are areas where the longitudinal and cross-sectional structures were less obvious and also appeared darker on micrographs. The sarcomere length in the area of dark shadows appeared shorter than in other areas indicating that dark shadow areas had contracted more and may be in an initial stage of a contraction band. In some dark shadow areas, Z lines could be recognized. Some longitudinal separations between myofibrils could be observed in non-shadow areas. The sarcomere length of the non-shadow area was 1.00μ. The short sarcomere length in non-shadow areas and the even shorter sarcomere length in dark shadow areas may explain the reduced tenderness of prerigor slow-heated samples.

In prerigor (0.5 hr postmortem) fast-heated (3.9°C/min) samples, many surface wrinkles (W) were obvious (Fig. 9) but did not continue around the complete circumference of the fiber. Figure 9 shows the surfaces of two fibers and the interior of one fiber. The distance between two wrinkles may range from 1 μ to 15 μ. Although the wrinkles on fiber surfaces of prerigor fast-heated samples looked similar to clefts on prerigor slow-heated samples, they differed in depth and frequency. Clefts tended to groove deeper but occurred less frequently. Surface bulges (B) appeared between wrinkles. Also, dark bands were visible in the cryofractured fiber interior. Dark bands were areas where individual structures were not distinguishable. Closer examination revealed dense packing in the dark band areas suggesting a severe contraction prior to or during heat denaturation. The severe contraction bands (C) were so intense that all structures seemed to fuse into a continuous mass. In the areas between contraction bands, myofibrils were disintegrated but recognizable. These areas seemed to be stretched (stretched areas, S) during formation of contraction bands. However, precise measurement of sarcomere length was not possible due to short, broken myofibrils in these areas and severe damage to intermyofibrillar connections. Contraction bands and stretched areas appeared to correspond to bright and dark stripes, respectively, in the phase contrast light micrographs (Fig. 3).

A very similar banding pattern was reported by Hsieh et al. (1980) using TEM. They showed many dense contraction bands

alternating with torn and fragmented areas on prerigor fast-heated bovine sternomandibularis muscle samples. However, the number of broken myofibrils in our study seemed much less than that of Hsieh et al. (1980).

There are other possible reasons for improved tenderness of prerigor fast-heated muscle samples in addition to broken myofibrils. Elevated pH values for prerigor cooked beef compared with postrigor cooked beef may improve tenderness. Bouton et al. (1973) reported that elevated pH lowered shear value and increased water-holding capacity of postrigor cooked beef. Although they did not specify the direct cause for shear force reduction, they presumed that increased water-holding capacity diluted the dry mass and reduced shear force. Another possible cause for reduced shear force of prerigor fast-heated samples may be damaged intermyofibrillar connections. As such connections serve for lateral force transmission for contracting muscle (Greaser, 1991), broken connections may reduce shear force.

Many features of our prerigor fast-heated samples were similar to those of thaw-rigor muscles reported by Cassens et al. (1963b). The dense, irregular, contracture bands they described were very similar to the bright stripes we found (phase contrast). The contracture bands on their TEM micrographs were similar to the contraction bands we found in prerigor fast-heated samples by SEM. Cassens et al. (1963a) reported similar features on several other violently treated muscles (e.g., cutting, fatal typhoid fever-caused muscular lesion, injection of boiling water and other irritants such as phenol and caffeine). Thus, collected evidence indicates that contractions caused by different stimuli may result in similar microstructural features, but only the most severe contractions lead to improved tenderness.

Closer examination of cryofractured interior of fibers (Fig. 9, upper right corner) reveals that the wrinkles on the surface correspond to interior stretched areas whereas surface bulges correspond to contraction bands. The contraction bands in one fiber appeared to continue into adjacent fibers (lower left corner). This occurred again on a cross-sectioned sample (Fig. 10). It also showed that the contraction bands did not occur circularly throughout the fiber. Instead, they occurred as channels from one surface point of the fiber to another surface point on the opposite side. These channels of contraction bands seemed to extend in a random manner rather than a straight form. The structural or metabolic cause for this unique structure was not clear.

CONCLUSION

SHORT POSTMORTEM DELAY TIME and fast heating rate may be combined in prerigor cooking of beef to give meat which is equal to or better than postrigor control beef in tenderness. Longer postmortem time (>6 hr) or slow heating rate (<1°C/min) resulted in reduced tenderness of prerigor cooked beef. Prerigor cooking might be used to improve tenderness of beef if postmortem delay time and heating rate were properly controlled.

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Dynamic Changes of Headspace Gases in CO_2 and N_2 Packaged Fresh Beef

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ABSTRACT

Two independent experiments were conducted to examine the effects of initial packaging/product conditions and storage conditions on in-package headspace pressure changes for modified atmosphere packaged beef during 12 hr storage. Headspace-to-meat volume ratio 1.8 to 5.9, surface area 200–800 cm^2, sample volume 0.22–0.75L, storage at 3–13°C, and initial gas composition 20–100% CO_2 balanced with N_2 were studied. Headspace-to-meat volume ratio was the most important packaging parameter, but surface area and meat volume also affected headspace CO_2 changes. Decreased storage temperature reduced CO_2 concentration remaining in headspace. Higher initial CO_2 concentration resulted in greater concentration changes.

Key Words: beef, headspace gases, modified atmospheres

INTRODUCTION

DEMANDS FOR ENHANCEMENT OF QUALITY and shelf-life of meats have increased applications of modified atmosphere packaging (MAP) of fresh meats. The in-package microenvironments of MAP meats are affected by dynamic changes of headspace gases (Seideman et al., 1979; Sebranek, 1986). Zhao et al. (1994) reviewed research on headspace CO_2 concentration changes during storage for CO_2/N_2 gas atmospheres, which indicated that meat absorption or evolution of CO_2 depended on initial headspace CO_2, temperature, packaging configuration, and meat characteristics. With high concentrations of CO_2 in the headspace of packaged meat, CO_2 would be absorbed by the muscle and fat tissue until saturation or apparent equilibrium resulted (Bush, 1991). Absorption of CO_2 causes a decrease in (headspace) volume in MAP resulting in shrunken or collapsed packages. Gill and Penney (1988) indicated that the effect of CO_2 addition in MAP would be complete only if CO_2 quantities were in excess of those required for meat saturation.

Models for prediction of gas concentrations in MAP systems for fresh produce have been based on Fick's Law of Diffusion to predict the rate of diffusion into the commodity (Arthur et al., 1989; Henig and Gilbert, 1975). Models analogous to those for fresh produce are not available for MAP meat. Furthermore, little quantative research has been reported on interactions among meat and gas headspace. Zhao (1993) developed a method to measure headspace CO_2 changes during storage of MAP fresh meats based on the ideal gas law, which enabled control and quantitative evaluation of product packaging configurations and MAP conditions created within a model package system. Results indicated that absorption was dependent upon size and shape of meat pieces, composition of exposed surfaces, boundary conditions at those surfaces (e.g., gas pressure and CO_2 concentration), and the ratio of headspace to meat volume (Gill, 1988; Taylor and MacDougall, 1973). Storage temperature and initial CO_2 concentration also directly affect CO_2 absorption (Spahl et al., 1981; Seideman et al., 1979; Enfors and Molin, 1984; Jackson, et al. 1992). Additionally, meat composition (e.g., moisture and fat content), pH, and other biological factors can greatly affect CO_2 absorption in packaged meat (Gill, 1988). Such relationships have not been expressed in precise mathematical terms.

Our specific objective was to quantify headspace CO_2 changes as related to product packaging configurations and storage conditions within the in-package modified atmosphere environment. The influence of packaging/product configuration on CO_2 absorption was modelled with a series of experiments conducted in a model package. The effect of storage conditions on CO_2 absorption coefficient was modelled with an experimental approach considering different storage temperatures and initial gas compositions.

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MATERIALS & METHODS

Model of CO_2 absorption coefficient

From the ideal gas law, headspace CO_2 changes with time can be related directly to headspace pressure change:

$$\frac{dn_{CO_2}}{dt} = \frac{44V_H}{R_g T}\frac{dP}{dt} \quad (1)$$

where dn_{CO_2}/dt is the amount of CO_2 change with time (g_{CO_2}/hr), V_H is the headspace volume of package, T is the temperature (K), R_g is the gas constant (8.3144 J/mole·K), and dP/dt is the headspace gas pressure change over time (kPa/hr). As with fresh produce (Arthur et al., 1989), CO_2 absorption coefficient on a mass/mass basis is then defined as:

$$R_{CO_2} = \left(\frac{dn_{CO_2}}{dt}\right)/M_m = \frac{V_{H/M}}{R_g T \rho_m}\frac{dP}{dt} \quad (2)$$

where R_{CO_2} is the CO_2 absorption coefficient ($g_{CO_2}/kg_{meat} \cdot hr$), $V_{H/M}$ is the headspace-to-meat volume ratio, and ρ_m is the density of the meat sample (kg/m^3).

Apparatus

A constant volume and gas impermeable chamber (255 mm diam by 110 mm high and 10 mm thick) was constructed of aluminum as a physical model of a MAP package. The change in CO_2 within the headspace of the model package was calculated from Eq. (1) by monitoring headspace gas pressure changes with time, using a pressure transducer (Omega Engineering Inc., PX 304-100AV) in the lid of the chamber connected to a strip chart recorder (ABB GOERZ Aktiengeschaft, Austria, Model SE120). Temperature inside the chamber was measured with a copper-constantan thermocouple probe (Omega Engineering Inc., Model CPSS-18G-8-RP) connected to the second channel of the strip chart recorder. Two metering valves attached to the chamber (Swagelok Co., Model SS-31RF2) were connected to a premixed gas bottle and vacuum pump (Thomas Industries Inc., Model 607CA32) for evacuation and gas back-flushing to replace the internal chamber atmosphere. A sampling port for withdrawal of gas samples and a pressure release valve were connected to the chamber.

The composition of headspace gases in the chamber was measured by removing 30 mL of gas via gas-tight syringe (Becton Dickinson and Company, Model B-D) through a shimadzu septum (Chemical Research Supplies, Model 77) attached to the sampling port. The volume percentage of individual gases was measured using a CO_2/O_2 gas analyzer (Servomex Co., Model 1450) to confirm the initial and final gas composition within the chamber during CO_2 absorption experiments.

The chamber volume was determined by the total volume of water that could be injected into the chamber (5.16L). The seal integrity of the

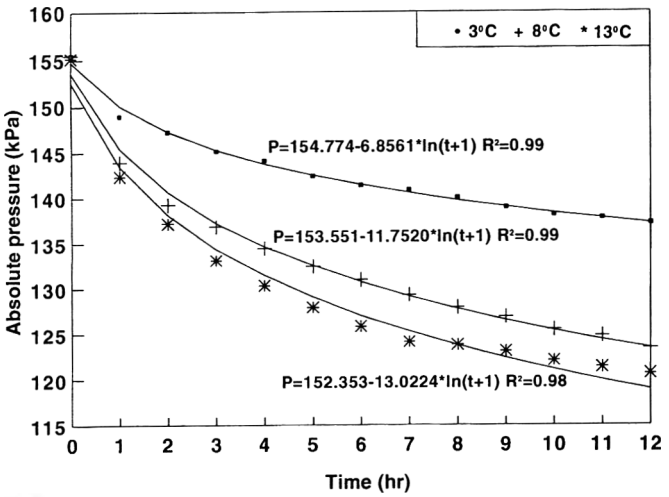

Fig. 1—Observed (symbols) and predicted (lines) time dependent pressure change for fresh beef stored at different temperatures with 100% CO_2 atmosphere.

chamber was determined by monitoring pressure change in nitrogen (initial gas pressure 207 kPa) during 24 hr. In order to achieve isothermal conditions, the test apparatus was located inside a temperature controlled chamber (Master-Bilt, Standex Company, Model D34LCD82) that was adjusted to the desired temperature 12 hr before the start of each experiment.

Preparation of meat samples

Semimembranosus muscles were obtained 72 hr postmortem either from the LSU Agricultural Center, Dept. of Animal Science (for packaging/product configurations) or a local commercial slaughter house (for storage conditions). Primal cuts were trimmed free of external fat and cut into rectangular pieces (samples) ranging from 260–860g, as required to adjust meat volume and surface area. All samples were initially vacuum packaged using a chamber-type, heat-seal vacuum packaging machine (Westglen Corp., Model VM 200H). Sample bags were numbered and stored at 4 ± 0.5°C for <7 days prior to beginning each experiment. Vacuum storage of samples prior to treatment exposure allowed for experimental control of variability anticipated due to differences in muscles between animals and within muscle variation for each animal. For each experiment, one beef sample was selected according to a previously assigned random order.

Proximate compositions of all samples were determined after testing using rapid analytical microwave procedures (CEM AVC-80 and Automatic Extraction System). The pH of each sample was measured with a glass body combination pH surface electrode attached to a digital pH meter (Oyster, Economy pH Meter, Model 301013-03-B). Various biological factors that could influence CO_2 absorption could not be controlled and were assumed constant for our experimental trials. All samples within an experiment were obtained from cattle with the same age and degree of finish, and were processed by a uniform procedure of slaughtering, chilling, and fabrication.

Packaging/product configuration

Beef samples (20) were tested to estimate the effect of headspace-to-meat volume ratio and surface area on CO_2 absorption. Headspace-to-meat volume ratios from 1.8 to 5.9 were established by varying chamber volume with addition of gas impermeable inserts and/or different meat sample volumes. Rectangular beef samples were chosen for ease of sample preparation and surface area measurement, with variations in surface area from 200 to 800 cm^2 and volume from 0.22 to 0.75L.

For each trial, a beef sample was placed in the test chamber maintained at 13 ± 0.5°C. We chose 13°C as a representative abusive storage condition for MAP meat. The chamber was sealed and connected to a vacuum pump and back-flushed using 100% CO_2. The vacuum/back-flush cycle was repeated three to four times until 99.0 ± 1.0% CO_2 had been reached. The initial headspace absolute gas pressure was adjusted to 155 ± 14 kPa by bleeding excess headspace gas through the metering valve. Initial CO_2 concentration was verified by gas analysis.

Each beef sample remained inside the chamber for a 12-hr monitoring period. Preliminary research indicated this 12-hr period was appropriate for monitoring headspace changes resulting in package collapse in high CO_2 atmosphere packages (Zhao, 1993). Pressure changes during storage were recorded and the CO_2 absorption (per unit mass of meat) was calculated.

Storage conditions

A 3 × 3 factorial, completely randomized design, with two replications (18 beef samples) was conducted to study time dependent headspace CO_2 changes (absorption/evolution) with three temperatures (3, 8, and 13°C) and 3 initial premixed gas compositions (100%CO_2:0%N_2, 50%CO_2:50%N_2, and 20%CO_2:80%N_2). During this experiment, all packaging/product parameters for beef samples were held constant ($V_{H/M} \approx 2.5$, $V_m \approx 0.34$L, and $S_m \approx 400$ cm^2).

During the experiment, each beef sample was placed in the chamber and maintained at the desired temperature. The atmosphere in the chamber was adjusted to that of premixed gases using the vacuum/back-flush procedure. The initial gas concentration was measured to verify the composition of commercially obtained premix gases. Final CO_2 and O_2 concentrations were analyzed and each beef sample was weighed after 12 hr inside the chamber. For each treatment combination, the CO_2 change in headspace was determined by monitoring the time dependent pressure change within the apparatus.

Statistical procedures

Experimental data from packaging/product configurations and storage conditions were analyzed by statistical regressions (SAS Institute Inc., 1989). Data for time dependent headspace gas pressure changes were used to develop pressure drop equations by least square regression using the SAS REG procedure. The statistical modeling technique of backward elimination was used to identify significant ($p < 0.05$) packaging/product and storage parameters that affected CO_2 absorption. Polynomial equations including significant parameters—headspace-to-meat volume ratio, surface area and volume of meat samples, as well as storage temperature and initial CO_2 concentration—were generated for CO_2 absorption values using the SAS general linear model REG procedure.

RESULTS & DISCUSSION

Pressure drop associated with CO_2 absorption

A function describing the time dependent pressure drop for CO_2 and N_2 packaged fresh beef was established using linear regression (SAS Institute Inc., 1989.) A logarithmic function described time dependent pressure:

$$P = \hat{P}_i - b \ln(t+1) \quad (3)$$

where t is the time (hr), $\hat{P}_i$ is initial absolute pressure of headspace gas as predicted by regression, and b is the initial pressure drop rate coefficient. The time dependent pressure drop fitted with Eq. (3) for beef muscle stored at 3, 8, and 13°C was compared under 100% CO_2 atmosphere (Fig. 1). The pressure drop for beef stored with 20%, 50%, and 100% CO_2 (balance of N_2) atmosphere at 3°C was also compared at 3°C (Fig. 2). Pressure drop model, Eq. (3), could be widely used to describe headspace pressure changes caused by CO_2 absorption under various storage temperatures and gas atmospheres (R^2 values for each regression line were 0.98 to 0.99). Since the initial pressure was constant for all experimental trials, the pressure drop rate coefficient b was related to different packaging conditions and intrinsic characteristics of the meat.

Therefore, CO_2 absorption coefficient could be related to initial pressure drop rate coefficient b by:

$$R_{CO_2} = \frac{44 V_{H/M}}{R_g T \rho_m} \frac{b}{(t+1)} \quad (4)$$

Eq. (4) indicates that CO_2 absorption coefficient is inversely proportional to time by the pressure drop rate coefficient (b) for conditions of $P_i \approx \hat{P}_i$. The larger the observed initial pressure drop rate, the more CO_2 absorbed by the meat tissue. Note that the CO_2 absorption would not continue unbounded, as the drop in CO_2 partial pressure within the headspace likely would reach some limiting threshold such that CO_2 would not diffuse across

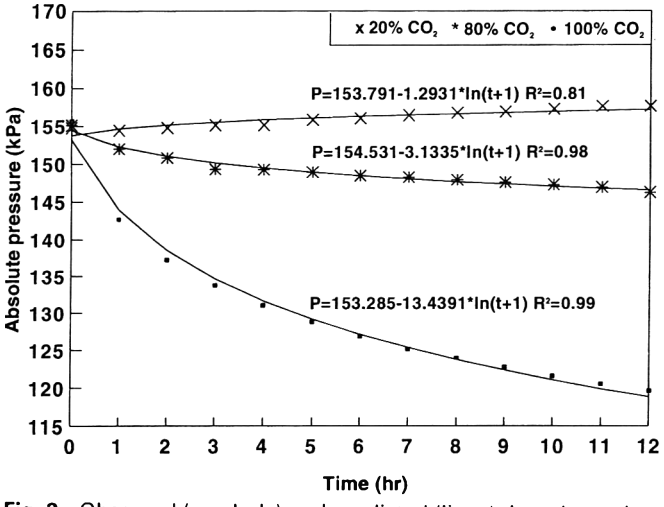

Fig. 2—Observed (symbols) and predicted (lines) time dependent pressure change for fresh beef packaged with different levels of CO_2 balanced with N_2 at 3°C.

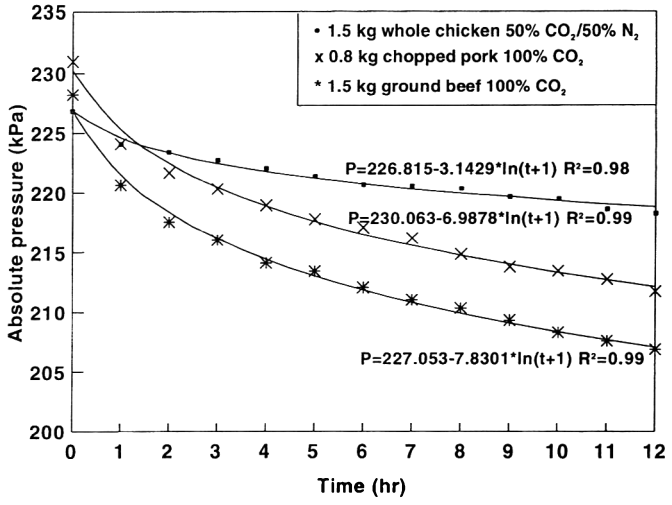

Fig. 3—Pressure change resulting from CO_2 absorption by chicken, pork, and beef at 13°C.

tissue membranes. This characteristic is reflected in the derivative of Eq. (3), dP/dt = b/(t + 1), indicating that dP/dt ≈ 0 after long time intervals. Figures 1 and 2 support the hypothesis that the rate of headspace gas pressure drop decreases with storage time. High pressure differential is the primary driving force for CO_2 absorption in beef tissue (Gill, 1988). As CO_2 is absorbed, the partial pressure (concentration) difference decreases, and the rate of headspace pressure drop would also decrease.

The CO_2 absorption during the first 12 hr of storage could be calculated:

$$A_{CO_2}(t=12)=\int R_{CO_2}\,dt=-\frac{44V_{H/M}}{R_g T \rho_m}\,b\,\ln(t+1)\Big|_{t=12} \qquad (5)$$

Where $A_{CO_2}(t = 12)$ is the accumulated amount of CO_2 absorbed (g/kg_{meat}) during 12 hr storage. Longer observation would likely reveal pressure changes associated with chemical conversions and microbial metabolic processes and not CO_2 absorption alone (Zhao et al., 1994).

Pressure drop with different meats

The general form of Eq. (3) was validated on studies with chicken, beef, and pork (unreported data). Examples of resulting pressure change data fitted by Eq. (3) were compared (Fig. 3). Results indicated that CO_2 absorption was related to type of muscle (i.e. different pressure curves reflect different CO_2 absorption of different muscles.) For example, a 1.5-kg whole chicken absorbed about 1.8 g of CO_2 in about 12 hr from an initial gas mixture of 50% CO_2 and 50% N_2. A 1.5-kg portion of ground beef and chopped pork absorbed about 4.5g and 5.3g of CO_2, respectively, at an initial 100% CO_2 gas atmosphere. Gill (1988) reported that CO_2 absorption in muscle tissue of pH 5.5 at 0°C was ≈ 960 mL (about 1.89 g/kg of tissue at STP) and that CO_2 absorption increased with increased tissue pH by 360 mL/kg for each pH unit, and decreased with increased temperature by 19 mL/kg for each 1°C rise. Bush (1991) reported that a whole chicken weighing 0.8-kg would absorb about 1.1g of CO_2 in 8 hr at equilibrium mixture of 50% CO_2 and 50% N_2, a 0.25-kg beef slice absorbed 0.4g of CO_2 in about 6 hr, and thin ham slices required only about 30 min under the same conditions to absorb the same amount of CO_2.

Effects of packaging/product parameters on CO_2 absorption

Equation (5) indicates that changes in headspace gas pressure directly reflect the amount of CO_2 absorbed by meat. The headspace pressure change caused by CO_2 absorption was related directly to the package/product configuration by the headspace volume (V_H) and mass of meat sample (M_m), and to package storage conditions by storage temperature (T). Other factors influencing CO_2 absorption could be indirectly related through functional correlation with the pressure drop rate coefficient (b). For beef the average initial moisture was 72.00 ± 2.05%, fat content 2.5 ± 2.0%, and pH was 5.5 ± 0.2%.

The pressure drop rate coefficient (b) values for 20 packaging/product configurations were compared (Table 1). Since headspace-to-meat volume ratio ($V_{H/M}$), surface area (S_m) and volume (V_m) of beef samples were assumed to be related to the pressure drop rate coefficient (b), a backwards elimination regression modeling technique was used to identify significant parameters. F-values observed with these packaging/product parameters were compared (Table 2). They indicate that the rate of headspace gas pressure changes caused by CO_2 absorption in fresh beef was related to headspace-to-meat volume ratio, surface area, and volume of beef sample, as well as interactions of those parameters.

Assuming S_m and V_m are constant, b could be approximated by the inverse of $V_{H/M}$:

$$b \approx b_{11}+\frac{b_{12}}{V_{H/M}} \qquad (6)$$

Equation (6) indicates that the initial pressure drop rate coefficient decreased with $V_{H/M}$ when $V_{H/M}$ was relatively small, but the effect of $V_{H/M}$ on b became small with increasing $V_{H/M}$ (Fig. 4). We hypothesized that there may be a critical point of $V_{H/M}$ such that a package would contain the minimum CO_2 in the headspace for meat to reach CO_2 absorption equilibrium. Empirically this could be further explained since a smaller $V_{H/M}$ would contain a smaller absolute amount of CO_2 within the headspace. When CO_2 in headspace is less than the minimum required to reach CO_2 absorption equilibrium (i.e. $V_{H/M}$ is relatively small), headspace gas pressure would decrease rapidly as meat tissue absorbed CO_2.

Since a critical $V_{H/M}$ value may reflect the minimum CO_2 available in the headspace to reach equilibrium, an increase in $V_{H/M}$ would represent excess CO_2 within the headspace such that the initial pressure drop rate would increase because of an increased concentration gradient. Thus, $V_{H/M}$ may be a critical package design parameter to achieve CO_2 absorption equilibrium for beef in 100% CO_2 microenvironment. Package collapse caused by CO_2 absorption in 100% CO_2 packaged meat may be avoided or minimized by increasing $V_{H/M}$ above a critical threshold (≈3.3 for the conditions observed).

Table 1—CO_2 absorption after 12 hr as related to packaging/product configuration of fresh beef (T = 13 ± 0.5°C, P_i = 155 ± 14 kPa, and H_i = 99 ± 1.0% CO_2 for all samples)

Sample size mm	M_m^a g	S_m^b cm^2	V_m^c L	$V_{H/M}^d$	b^e kPa/hr	A_{CO_2} (t=12)^f g/kg_{meat}
164×118×29	643.6	550.6	0.5363	1.797	10.88	0.8404
165×132×23	638.2	572.2	0.5318	1.820	11.32	0.9540
180×135×23	642.8	630.9	0.5357	2.435	8.99	0.9241
140×124×26	520.6	484.5	0.4338	2.458	9.00	0.9195
150×148×25	660.4	593.0	0.5730	2.867	6.86	0.8458
150×145×25	655.0	582.5	0.5683	2.899	8.45	0.8471
180×170×24	868.4	780.0	0.7503	3.165	5.23	0.7016
180×100×42	849.8	595.2	0.7342	3.256	4.68	0.6677
180×170×24	848.0	780.0	0.7327	3.265	5.08	0.7238
180×100×40	841.0	584.0	0.7269	3.299	5.20	0.7426
170×90×50	813.6	566.0	0.7059	3.427	4.23	0.6294
150×90×30	437.0	414.0	0.3642	4.052	5.93	0.9964
180×110×30	717.0	570.0	0.5976	4.229	8.18	1.1531
140×115×17	410.4	408.7	0.3421	4.379	6.13	1.1237
160×110×33	613.8	530.2	0.5116	5.108	5.69	1.1743
160×110×20	360.4	460.0	0.3001	5.130	6.75	1.3977
125×90×20	284.6	211.0	0.2372	5.324	7.13	1.6222
128×83×20	264.2	296.6	0.2202	5.812	8.42	1.9880
144×110×15	323.8	393.0	0.2699	5.817	6.59	1.5986
142×120×22	491.4	456.1	0.4095	5.899	4.74	1.1035

[a] M_m equals initial mass of individual samples.
[b] S_m equals measured surface area of the sample.
[c] V_m equals volume of each sample.
[d] $V_{H/M}$ equals headspace to meat volume ratio.
[e] b equals initial pressure drop rate.
[f] A_{CO_2} equals amount of CO_2 absorbed per unit mass of meat.

Table 2—F values for significant variables for the headspace pressure drop rate coefficient

Variable	Regression coefficients[b]	F value[a]
Intercept	9.886	19.70 (0.0008)
$1/V_{H/M}$	−37.440	14.67 (0.0024)
S_m	−186.753	17.00 (0.0014)
V_m	−23.975	13.09 (0.0035)
$S_m/V_{H/M}$	838.784	17.92 (0.0012)
$V_m/V_{H/M}$	86.394	12.97 (0.0036)
S_m/V_m	467.332	14.67 (0.0024)
V_mS_m/V_m	−1803.865	15.91 (0.0018)

[a] F-value (probability of a large number of F)
[b] Regression coefficients are given for the mathematical model used for this statistical analysis:

$$b = b_0 + \frac{b_1}{V_{H/M}} + b_2S_m + b_3V_m + b_4\frac{S_m}{V_{H/M}} + b_5\frac{V_m}{V_{H/M}} + b_6V_mS_m + b_7S_m\frac{V_m}{V_{H/M}}$$

Coefficient of determination for this model R^2 = 0.91

The F-values for A_{CO_2}(t = 12) with packaging/product parameters were obtained by the elimination of variable method (Table 3). They indicate that in addition to $V_{H/M}$, 12 hr CO_2 absorption was related to surface area, volume of beef sample, and interactions of these parameters. The significant F-value for S_m and $V_{H/M} \times S_m$ showed that the surface area of a beef sample affected 12 hr CO_2 absorption, but the influence of S_m on A_{CO_2}(t = 12) was dependent on $V_{H/M}$. This indicates that the larger the surface area, the higher would be the 12 hr CO_2 absorption. Since surface area can vary with geometry for a given volume or mass of meat, selection of suitable geometry for meat in the design of a MAP system can assist in controlling CO_2 absorption. For example, to achieve CO_2 absorption equilibrium quickly, a thin and long meat sample should be used instead of a relatively thick sample with the same volume.

While absorption is influenced by beef sample volume, it is also related to meat surface area and headspace-to-meat volume ratio as indicated by significant F-values for $V_m \times V_{H/M} \times S_m$ (Table 3). This indicates that CO_2 absorption in packaged meat is not strictly a surface phenomenon, but also that CO_2 diffused and penetrated inside the meat. However, since the volume of beef samples was difficult to control and limited, the quantitative effect of V_m and relationship of V_m to S_m and $V_{H/M}$ was restricted. Further investigation with a controlled meat sample volume is needed to determine how CO_2 absorption is affected by V_m.

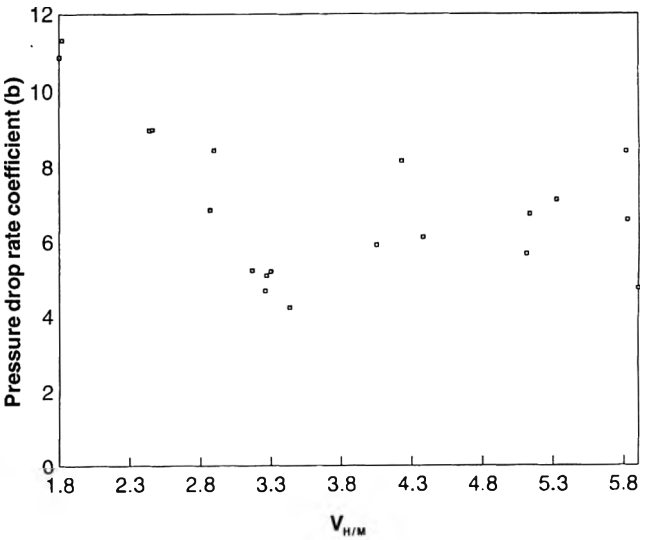

Fig. 4—Pressure drop rate coefficient as related to headspace-to-meat volume ratio for fresh beef.

Table 3—F values for significant variables for 12 hr CO_2 absorption

Variable	Regression coefficients[b]	F value[a]
Intercept	−8.582	32.67
$V_{H/M}$	2.403	54.80
S_m	194.382	37.16
V_m	20.424	34.99
$V_{H/M} \times S_m$	−44.497	41.02
$V_m \times S_m$	−421.365	9.47
$V_{H/M} \times V_m$	−5.848	37.66
$V_{H/M} \times V_m \times S_m$	111.525	38.23

[a] F-value (all probabilities were 0.0001).
[b] Regression coefficients are given for the mathematical model used for this statistical analysis:

$$A_{CO_2} = a_0 + a_1V_{H/M} + a_2S_m + a_3V_m + a_4V_mV_{H/M} + a_5V_{H/M}S_m + a_6V_mS_m + a_7V_mV_{H/M}S_m$$

Coefficient of determination: R^2 = 0.97

Effects of storage conditions on headspace CO_2 changes

Data on the effects of storage temperature and initial CO_2 concentration on amounts of CO_2 changes in 12 hr were summarized (Table 4). The measure "amount of CO_2 change" instead of "CO_2 absorption" was used because CO_2 evolution from beef tissues was observed when gas mixtures of CO_2 and N_2 were used. The net action of absorption and evolution was calculated as the final amount of CO_2 change in the headspace.

The 12 hr CO_2 changes as related to temperature were compared for each initial CO_2 concentration (Fig. 5). For an initial 100% CO_2 package, headspace CO_2 absorption decreased with increased temperature. Within the range 3–13°C, the amount of CO_2 change decreased linearly by ≈0.02 g/kg_{meat} (about 10.2 mL/kg_{meat} at standard conditions) for each 1°C rise (Fig. 5). For an initial atmosphere of 50% CO_2/50% N_2, 12 hr amount of CO_2 change was almost parallel with the 100% CO_2 line, and temperature effect on CO_2 absorption was almost the same as the 100% CO_2 atmosphere. When an initial atmosphere of 20% CO_2 plus 80% N_2 was used, the 12 hr CO_2 change appeared negative (Fig. 5). This was because additional CO_2 was evolved from beef tissues in excess of that absorbed, even at lower temperatures. The higher the storage temperature, the more CO_2 evolved from the beef.

The initial CO_2 concentration in headspace greatly affected the dynamic conditions inside the package microenvironment. The major factors that influence the amount of CO_2 changes include CO_2 absorption in meat, diffusion of CO_2 from preformed CO_2 pools within the meat, aerobic energy metabolism of meat cells, and other biochemical reactions (Enfors and

Table 4—CO_2 absorption after 12 hr as related to storage conditions of fresh beef ($V_{H/M}$=2.5, S_m=400 cm^2, and V_m=0.34 L for all samples)

Temp °C	Initial MAP CO_2%	N_2%	P_i^a kPa	P_f^b kPa	M_m g	A_{CO_2} (t=12) g_{CO_2}/kg_{meat}
3	100	0	155.69	119.63	395.8	1.5235
			155.48	116.87	396.6	1.5873
	50	50	155.44	146.17	402.8	0.3700
			155.34	138.73	389.0	0.6963
	20	80	154.79	157.55	395.6	−0.1134
			154.79	155.34	398.2	−0.0220
8	100	0	155.48	123.42	351.4	1.3310
			155.14	118.59	390.6	1.4756
	50	50	155.14	147.90	401.0	0.3004
			154.45	141.00	392.6	0.5429
	20	80	155.14	157.90	393.2	−0.1454
			155.14	158.24	394.4	−0.1296
13	100	0	154.10	123.08	399.0	1.2656
			155.14	120.66	386.6	1.4147
	50	50	155.14	147.90	329.6	0.2953
			154.79	144.80	389.2	0.4102
	20	80	155.14	166.86	383.8	−0.4779
			155.14	162.93	385.8	−0.3183

[a]P_i equals Initial headspace gas absolute pressure.
[b]P_f equals Headspace gas absolute pressure at the end of 12 hr.

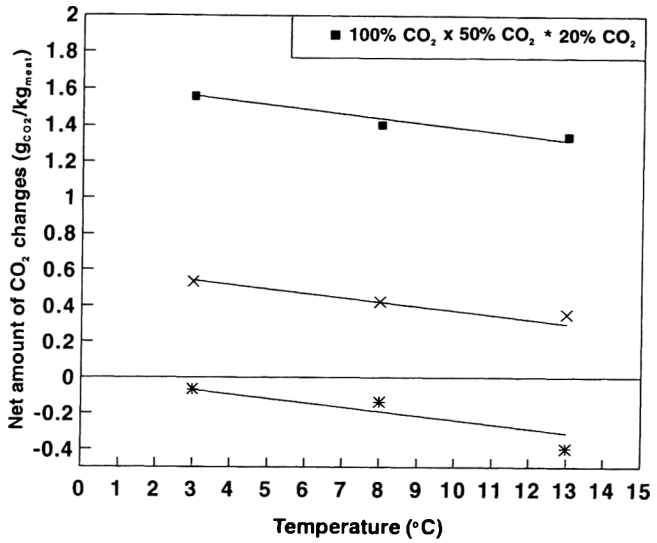

Fig. 5—Headspace CO_2 changes as related to storage temperatures for 12 hr storage of fresh beef.

Molin, 1984; Gill, 1988). For an initial 100% CO_2 package, respiration and other aerobic energy metabolism are considered to be inhibited by a high concentration of CO_2 in headspace. Since CO_2 is highly soluble in both water and oil, it would be absorbed by the muscle and fat tissues until apparent equilibrium. When a gas mixture of 50% CO_2/50% N_2 was used in headspace, CO_2 absorption still was important headspace gas changes. However, the amount of CO_2 absorbed by beef was much less than 100% CO_2 package (Fig. 2). When the initial CO_2 concentration was decreased to 20%, the amount of CO_2 evolved was in excess of that absorbed by meat. Therefore, the final amount of CO_2 in the headspace increased, appearing as a negative amount of CO_2 absorbed.

A headspace change model, Eq. (5) was applied to the 12 hrs amount of CO_2 change using the SAS REG procedure:

$$A_{CO_2}(t=12) = 6.203158 - 0.024193\ T + 0.020377\ H_i \quad (7)$$

where $A_{CO_2}(t = 12)$ is the 12 hr amount of CO_2 change, expressed as a linear function of T, the absolute temperature (K), and H_i, the initial CO_2 concentration (%). No significant interactions between temperature and initial CO_2 concentration were noted. The coefficient of determination for Eq. (7) was 0.97.

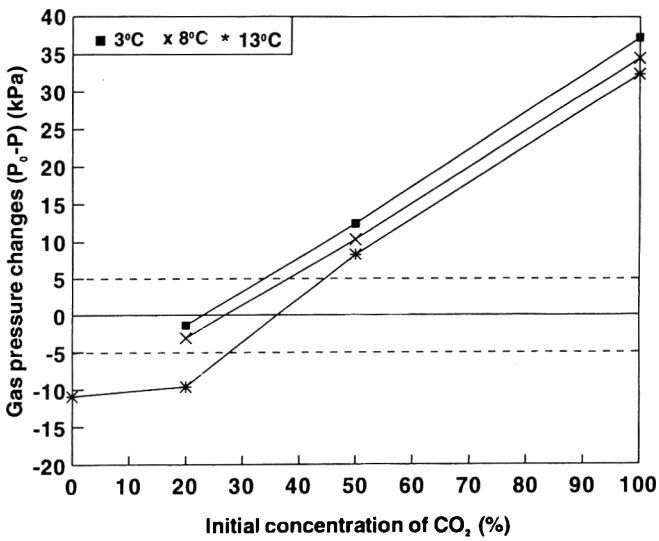

Fig. 6—Pressure changes in headspace as related to storage temperatures and initial CO_2 concentrations for 12 hr storage of fresh beef.

Engineering design criteria for MAP packaged meat

Meat composition (e.g., moisture and fat content), pH and other biological factors can greatly affect CO_2 absorption in packaged meat (Gill, 1988). Quantative determination of the effects of packaging/product configuration on CO_2 absorption would require a comprehensive study to precisely elucidate the many factors involved. Our results show that headspace-to-meat volume ratio is an important packaging parameter on 12 hr CO_2 absorption while surface area and volume of beef sample were less influential. The specific influence of beef sample volume on this conclusion is unclear.

In flexible packaging systems the headspace gas pressure influences the external appearance. A headspace pressure less than atmospheric causes collapse, whereas pressure higher than atmospheric would result in swollen and possibly broken packages. For each storage temperature there is an initial gas composition range that could minimize headspace gas pressure changes within a narrow range (almost constant) during storage (Fig. 6). For example, an initial concentration of 28% to 45% CO_2 balanced with N_2 within a package could achieve stable headspace gas pressure (0 ± 5.0 kPa) at 13°C (Fig. 6). The initial concentration of CO_2 required to achieve stable headspace pressure is related to temperature. The lower the storage temperature, the lower the initial CO_2 concentration needed to achieve constant headspace gas pressure. Furthermore, packaging parameters, such as headspace-to-meat volume ratio and surface area and volume of meat sample, are important factors in determining headspace gas pressure changes.

CONCLUSIONS

IN MAP HEADSPACE-TO-MEAT VOLUME RATIO is the most important factor influencing CO_2 headspace changes. Surface area and volume of a meat sample also influence changes in headspace CO_2. Temperature and initial gas composition greatly affect CO_2 headspace changes. When initial CO_2 percentage was >35% (balanced with N_2) for product at 13°C, the net absorption of headspace CO_2 was observed in packaged meat, otherwise, net CO_2 evolution occurred. Both CO_2 absorption and evolution were influenced by storage temperature. Higher temperatures reduced CO_2 absorption, while increasing CO_2 evolution.

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Composition and Chemistry of Mechanically Recovered Beef Neck-Bone Lean

B.P. DEMOS and R.W. MANDIGO

ABSTRACT

The objective was to characterize basic chemical and functional differences among mechanically recovered neck-bone lean beef (MRNL), hand-trimmed neck-bone lean beef, a control of 85% beef lean trim and bone marrow. MRNL had higher ($P<0.05$) metmyoglobin reducing ability and more total iron than the control and more fat ($P<0.05$) than hand trim. Values for pH were higher ($P<0.05$) for MRNL than for hand trim or the control (6.68, 6.33 and 5.80, respectively). MRNL also had higher ($P<0.05$) nonheme iron, total pigment and water-holding capacity. Bone marrow had higher ($P<0.05$) ash, cholesterol, total iron, total pigment and pH than any lean types.

Key Words: beef, neck bone, bone marrow, metmyoglobin, water-holding

INTRODUCTION

MECHANICAL SYSTEMS that recover lean tissue from split beef cervical vertebrae portions have been developed. This technology has led to questions concerning functional differences in tissue obtained by this process vs lean tissue obtained by hand-trimming. Mechanically recovered neck bone lean (MRNL) from intact beef neck bones has altered functional properties that may provide advantages as a raw material in processed meat products. Such altered properties include higher pH and metmyoglobin reducing ability that could result in higher water-holding capacity and greater color stability. Many differences have been documented for various types of mechanically deboned meat, such as pH (Anderson and Gillett, 1974), pigment content (Field et al., 1980), mineral content (Field et al., 1974) and amino acid profile (Field et al., 1978; Golan and Jelen, 1979). Sensory and physical differences of processed products containing mechanically deboned meat have been shown (Joseph et al., 1978; McMillin et al., 1980; Ockerman et al., 1981; Defreitas and Molins, 1991). Such differences, if found for MRNL from split beef cervical vertebrae, would provide a basis for increased value of MRNL. Research may identify potential processing benefits from utilization of MRNL in higher-valued products. Areas of interest include improvements in color, water-holding capacity and textural attributes.

Much previous research involved lean that was recovered from bones ground and forced through a small aperture to separate chips from salvageable lean meat (Anderson and Gillett, 1974; Joseph et al., 1978; McMillin et al. 1980) or mixtures of lean meat salvaged from different portions of a carcass (Joseph et al., 1978; Mott et al., 1982). Our objective was to study lean beef recovered from split beef cervical vertebrae and to identify any differences in functional properties.

MATERIALS & METHODS

Manufacture of beef neck bone lean

Untrimmed beef neck bones (3,128 kg, 865 bones) from production at a commercial meat plant were obtained 3 days postmortem (stored at 4°C). Bones were sawed to conform to a Protecon PAD 400 (Stork Protecon, Inc., Gainesville, GA) automatic trimmer. The atlas bone was removed. Feather bones were removed closely along the top ridge of neck bones. The neck bones were cut to length (54.6 cm) at the posterior end. Total trimmed weight of the neck bones was 2,059 kg.

Pressed lean from a Protecon PAD 400 trimmer was processed through a Baader Lean Separator (Baader North America Corp., New Bedford, MA). This separator processes intermediate material between a specially designed neoprene belt and a drum-screen configuration that is effective in removing sinews, tendons, connective tissue and notable bone chips. The Baader machine was set up in two different configurations. Batch one of the intermediate pressed lean was processed with the Baader meachine in a desinewing configuration at 40 bar pressure (Baader 1 lean). The remaining lean was processed with the Baader machine in a deboning set-up at 30 bar pressure (Baader 2 lean). A small portion of intermediate pressed lean was not processed and was saved for analysis (Protecon lean).

All lean was packaged in plastic bags, placed in wax-lined cardboard boxes and frozen at −35°C. Each bag weighed ≈ 4.5 kg. Several untrimmed neck bones were vacuum-packaged, placed in wax-lined boxes and frozen at −35°C until further analysis.

Sampling

Three replications were performed. For each replication, three frozen bags of each type of Baader lean (Baader 1 or 2) and one bag of intermediate lean were removed from the freezer and a 2.54-cm center slice of each bag was removed with a band saw. The center slices for each type of lean were cut into 2- to 3-cm^2 pieces, frozen in liquid nitrogen and powdered in a Waring blender. The powder was placed in doubled plastic bags and stored in an ultra-low freezer (Revco Scientific, Inc., Asheville, NC) at −80°C.

For each replication, two untrimmed neck bones were hand-trimmed with a Bettcher 520 whizzard knife (Bettcher Industries, Inc., Vermillion, OH). Bone marrow was then extracted from the trimmed bones according to the method of Field et al. (1978). Bone cortex from the vertebrae was removed with a band saw and the spongy bone was cut into pieces of ≈ 2 × 2 cm. Pieces of spongy bone were placed on perforated platforms inside centrifuge tubes and spun a 17,000 × g for 30 min to separate marrow from spongy bone. Ground beef controls (≈ 85% lean) were purchased from a local meat retailer for each replication. All hand trim, marrow and control samples were powdered and stored as described.

Chemical analysis

Analyses of fat, protein, moisture and ash were carried out by methods of the AOAC (1990). The method of Oles et al. (1990) was used to measure cholesterol. Total iron content was determined by atomic absorption spectrophotometry as outlined by the Perkin-Elmer Corporation (1964). Nonheme iron was measured by the method of Rhee and Ziprin (1987).

Total pigment determination

Total meat pigment was measured by a modified method of Karlsson and Lundstrom (1991). Powdered sample (5g) was weighed into 250-mL bottles and kept on ice. Samples were homogenized with a Polytron homogenizer (Brinkman Instruments, New York, NY) set at speed setting 4 (10,800 rpm), after adding 50 mL of 0.5 M phosphate buffer, pH 7.4. The bottles were capped and stored overnight at 4°C in the dark. Samples were stirred and filtered through Whatman No. 42 paper. To 4 mL of filtrate were added 0.4 mL of 10% Triton X-100 detergent solution and 0.25 mL of 5 M NaOH and mixed. Absorbance was read at 575 and 700 nm (Gilford Response spectrophotometer, Gilford Instruments Laboratories, Oberlin, OH).

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Table 1—Chemical analysis of types of lean beef and bone marrow

	Protecon[a]	Baader 1[b]	Baader 2[c]	Hand trim	Marrow	Control	SEM
Moisture (%)	66.60[d]	66.02[d]	67.72[de]	70.98[f]	69.84[ef]	66.33[d]	1.11
Fat (%)	16.57[d]	17.34[d]	16.14[d]	10.55[e]	15.83[d]	14.97[de]	1.50
Protein (%)	17.17[e]	17.02[e]	16.94[e]	20.85[d]	14.25[f]	19.86[d]	0.60
Ash (%)	0.88[d]	0.76[d]	0.90[d]	0.87[d]	1.55[e]	0.83[d]	0.06
Cholesterol (mg/g meat)	1.56[f]	1.03[de]	1.15[e]	0.86[de]	2.08[g]	0.62[d]	0.11
Nonheme iron (µg/g meat)	7.53[e]	15.00[d]	14.96[d]	5.23[ef]	13.51[d]	3.02[f]	1.02
Total iron (mg/100g meat)	4.93[d]	6.61[d]	6.77[d]	2.26[e]	13.01[f]	1.58[e]	0.64
Total pigment (mg/g meat)	13.26[e]	15.60[e]	17.16[e]	7.02[f]	41.86[d]	5.98[f]	1.82

[a] Protecon = Pressed lean, before being processed through the Baader Lean Separator.
[b] Baader 1 = Pressed lean processed through the Baader Lean Separator in a desinewing configuration.
[c] Baader 2 = Pressed lean processed through the Baader Lean Separator in a deboning setup.
[defg] Means in the same row with unlike superscripts differ ($P<0.05$).

To determine hematin concentration, a standard curve of hematin chloride solutions was prepared. A stock solution of hematin chloride dissolved in the phosphate buffer, 10% Triton X-100 detergent and 5M NaOH, in the same proportions as found in the samples, was made. This solution was diluted so the standard curve would include concentrations of interest. Absorbance of all standard curve solutions was read at 575 and 700 nm. A regression equation to regress hematin concentration on A_{575}-A_{700} was calculated. The equation, averaged across the 3 replications, was:

$$\text{ppm hematin} = 12.9 \times [96.715 \times (A_{575}\text{-}A_{700}) - 0.531] \; (R^2=0.99)$$

Parts per million hematin were converted to mg of meat pigment per g of tissue (wet wt) with the 0.026 conversion factor of Franke and Solberg (1971).

Measurement of pH

Powdered samples (10g) were homogenized in 100 mL of distilled/deionized water. The pH was measured on duplicate samples from each type of lean with a Corning general purpose electrode (Corning Glass Works, Corning, NY).

Water-holding capacity

Water-holding capacity was measured by a modified method of Wierbicki and Deatherage (1958). A 400- to 600-mg sample was weighed on Whatman No. 1 filter paper. The sample and paper were placed between plexiglas plates and pressed in a Carver laboratory press (Fred S. Carver, New York, NY) at 6,895 kPa for 1 min. Areas of the juice ring and the pressed sample were measured with a digital planimeter (Planix 6, Tamaya Technics, Inc., Tokyo, Japan). Results were expressed as percentage total moisture content that was free water according to the equation of Wierbicki and Deatherage (1958):

$$\text{Percent free water} = \frac{(\text{total area} - \text{meat film area}) \times 61.10}{\text{total moisture (mg) in sample}} \times 100$$

Metmyoglobin reducing ability

Metmyoglobin reducing ability (MRA) was measured by a modified method of Ando and Nagata (1970). Powdered sample (7g) and 14 mL of pH 5.5 veronal buffer [5 parts veronal acetate solution (19.4g sodium acetate crystals, 29.4g sodium diethyl barbiturate made up to 1L with dd H_2O), 8 parts 0.1M HCl, 12 parts dd H_2O], were homogenized for 10 sec with a Polytron homogenizer (Brinkman Instruments, New York, NY) at speed setting 4 (10,800 rpm). The homogenate was then diluted to 50 mL with distilled/deionized water. Diluted sample (5 mL) and 2 mL of 5 mM potassium ferricyanide solution were mixed and allowed to stand at 0°C for 1 hr with occasional stirring. Ammonium sulfamate 0.1 mL of 0.04 M solution, and 0.2 mL of 0.5 M lead acetate solution were added and the mixture was allowed to stand for 10 min at room temperature (≈23°C). Before making solutions up to 10 mL with distilled/deionized water, 2.5 mL of 20% trichloroacetic acid solution was added. Mixtures were filtered through Whatman No. 4 paper, and absorbance of filtrate was measured at 420 nm (Gilford Response spectrophotometer, Gilford Instruments Laboratories, Oberlin, OH).

A 1 mM potassium ferricyanide solution was mixed and its absorbance was read at 420 nm. Metmyoglobin reducing ability was calculated as the difference between absorbance of the 1 mM potassium ferricyanide solution and absorbance of the observed sample. Higher values are indicative of greater MRA.

Oxidation-reduction potential

Powdered sample (10 g) and 15 mL of 0.1 M phosphate buffer (pH 6.0) were placed in a Waring Blendor. A rubber hose was sealed in a hole cut in the blender lid. The hose was connected to a vacuum pump to minimize oxygen incorporation. Sample and buffer were homogenized 15 sec. Oxidation-reduction potential was measured with a redox combination electrode (Corning Glass Works, Corning, NY) attached to a pH meter after 2 min equilibration.

Statistical analysis

Data were analyzed by the General Linear Models procedure of SAS (1988) as a randomized complete block design, with replication used as the blocking criterion. Means were compared by least significant difference if a significant F-ratio ($P<0.05$) was observed for the appropriate source of variation.

RESULTS & DISCUSSION

INITIAL WEIGHT was 3,128 kg of whole untrimmed beef neck bones. After sawing to conform to the Protecon PAD 400 mold, weight of the neck bones was 2,059 kg. Total lean yield after passing through the Protecon automatic trimmer was 319 kg. Temperature rise after passing the intermediate pressed lean through the desinewing Baader set-up (Baader 1) was 9°C and yield was 86.4%. Temperature rise with the Baader machine in the deboning set-up (Baader 2) was 1°C and yield was 90.5%.

Chemical analyses of the various types of lean were compared (Table 1). Hand trim had higher ($P<0.05$) moisture than all treatments except marrow. No differences ($P>0.05$) occurred in moisture among intermediate Protecon lean, Baader 1 lean, Baader 2 lean or the control. Hand trim had lower ($P<0.05$) fat than Protecon lean, Baader 1 lean, Baader 2 lean or marrow, but no difference ($P>0.05$) occurred in fat between hand trim and control. Protein among Protecon lean, Baader 1 lean or Baader 2 lean was not different ($P>0.05$) and all three had lower ($P<0.05$) protein than hand trim or control which were not different ($P>0.05$) from one another. Marrow had lower protein than all other treatments ($P<0.05$). Ash was not different ($P>0.05$) among all treatments except marrow which had greater ($P<0.05$) ash than all others.

In a similar study with mutton, Anderson and Gillett (1974) found mechanically deboned lean (whole shoulder and neck ground prior to lean recovery) contained more ($P<0.05$) moisture and ash and less ($P<0.05$) protein and fat than hand-deboned lean from the same carcass parts. Ockerman et al. (1981) found mechanically deboned lean from pork vertebrae had higher moisture and ash and lower protein than hand-deboned lean from pork vertebrae. They also found fat content was similar. Comparisons across species for proximate composition may

Table 2—Metmyoglobin reducing ability, oxidation-reduction potential, pH and water-holding capacity of types of lean beef and bone marrow

	Protecon[a]	Baader 1[b]	Baader 2[c]	Hand trim	Marrow	Control	SEM
Metmyoglobin reducing ability (unitless)	0.34[de]	0.39[ef]	0.41[ef]	0.37[de]	0.49[f]	0.29[d]	0.04
Oxidation-reduction potential (mV)	210.7[d]	218.5[d]	220.4[d]	192.5[e]	212.2[d]	220.5[d]	3.42
pH	6.64[e]	6.66[e]	6.70[e]	6.33[f]	7.71[d]	5.83[g]	0.03
Water-holding capacity (% free water)	41.90[d]	33.97[g]	31.49[h]	39.03[e]	—	36.24[f]	0.59

[a] Protecon = Pressed lean, before being processed through the Baader Lean Separator.
[b] Baader 1 = Pressed lean processed through the Baader Lean Separator in a desinewing configuration.
[c] Baader 2 = Pressed lean processed through the Baader Lean Separator in a deboning setup.
[defgh] Means in the same row with unlike superscripts differ ($P<0.05$).

not be valid. Probably method of mechanical lean recovery affects proximate composition. When mechanical pressure is used to force lean away from vertebrae and through small apertures, some components probably occur in different proportions than found in hand trim. The pressing and separating processes as well as presence of various materials not normally found in lean trim (such as spinal cord and bone marrow) are likely causes of such differences.

Cholesterol content was not different ($P>0.05$) among Baader 1 lean, hand trim or control; however, Baader 2 lean had a greater ($P<0.05$) cholesterol content than the control. Marrow from the neck bones was probably pressed into the lean meat during mechanical recovery, which caused cholesterol content of the Baader lean to be high. Cholesterol was lowered after the Protecon lean was passed through the Baader machine.

No differences ($P<0.05$) were found among Baader 1 lean, Baader 2 lean or bone marrow for nonheme iron content. All were higher ($P<0.05$) in nonheme iron than Protecon lean, hand trim or control (Table 2). Hand trim was not different ($P>0.05$) than Protecon lean or the control for nonheme iron content; however, the control was lower ($P<0.05$) in nonheme iron than Protecon lean. Increased nonheme iron content of Baader 1 and Baader 2 lean was probably due to bone marrow. It contains high concentrations of two nonheme compounds, hemosiderin and ferritin (Underwood, 1971). Note that the nonheme iron increase was not so much due to pressing action of the Protecon automatic trimmer, but rather to bone marrow being incorporated during processing (Table 1). A negative implication is that nonheme iron is a major catalyst of oxidation in cooked meats (Igene et al., 1979; Love, 1983). This could be a potential problem if Baader lean was used in pre-cooked processed meat items.

No differences occurred ($P>0.05$) for total iron content between hand trim and control, and both were lower ($P<0.05$) in total iron than all other lean types and bone marrow (Table 2). No differences ($P>0.05$) occurred among Protecon lean, Baader 1 lean and Baader 2 lean for total iron. Bone marrow was higher ($P<0.05$) in total iron than all types of lean. Increased total iron levels were apparently due to marrow incorporation during the recovery process.

Total pigment concentration was closely related to total iron ($r = 0.85$, $P<0.01$). Hand trim and the control were lower ($P<0.05$) in total pigment than all other lean types or bone marrow (Table 1). Bone marrow had more ($P<0.05$) total pigment than all lean types. Ahn and Maurer (1989) reported that color of fresh meat varied with amount of pigment present. If the increased pigment found in Baader 1 and Baader 2 lean could be kept in the reduced oxymyoglobin form a longer time than controls (as suggested by MRA and visual observation), the opportunity would exist to improve consumer appeal of ground beef. By incorporating such lean sources into a ground beef system, it may be possible to create a more intense cherry red color with extended life at the retail level.

Bone marrow had a higher ($P<0.05$) pH than all types of lean beef. No difference occurred ($P>0.05$) in pH among Protecon lean, Baader 1 lean or Baader 2 lean. However, all three lean types had a higher ($P<0.05$) pH than hand trim or control, due to marrow incorporation. Similar results with mechanically deboned meat (bones ground prior to lean retrieval) were reported by Anderson and Gillett (1974). Field and Arasu (1981) showed significant increases in pH of ground beef with increased addition of bone marrow. They reported a correlation of 0.96 between marrow concentration and pH.

Each lean type was different ($P<0.05$) from all other lean types or bone marrow for water-holding (WHC) capacity (Table 2). The order from highest to lowest WHC was Baader 2 lean > Baader 1 lean > control > hand trim > Protecon lean. Note that Baader 1 and Baader 2 lean had greater ($P<0.05$) WHC than hand trim or control. This could be of particular importance if Baader 1 or Baader 2 lean were used in a coarse ground or emulsified processed meat formulation. McMillin et al. (1980) showed increased ($P<0.05$) WHC with increased levels of mechanically recovered pork in an emulsified product. They suggested that it was due to availability of more polar groups of proteins due to size reduction of meat particles. Our WHC results confirmed this, since Baader 1 and Baader 2 lean underwent the greatest particle reductions. Increased WHC might also be attributable to increased pH in Baader 1 and Baader 2 lean. However, Protecon lean was not different ($P>0.05$) from Baader 1 or Baader 2 lean in pH, but it was lower in WHC than all lean types.

No difference occurred ($P>0.05$) in MRA among Protecon lean, Baader 1 lean, Baader 2 lean or hand-trim (Table 2). Baader 1 lean and Baader 2 lean had a greater ($P<0.05$) MRA than control. Bone marrow had a greater ($P<0.05$) MRA than Protecon lean, hand-trim or control. Note that Baader 1 and Baader 2 lean had a higher MRA than control. This type of recovered lean might provide better color stability in a ground beef product. Ledward (1985) suggested reduction of metmyoglobin (metMb) prolongs color stability by reducing metMb to myoglobin, and that enzymatic MRA was the most important factor in determining the oxidation state of myoglobin. In the presence of oxygen, myoglobin is converted to oxymyoglobin, which is bright cherry red. This may explain visual observations we made. Baader 1 and Baader 2 lean appeared to retain the bright cherry red color of oxymyoglobin much longer than hand trim or control. Hutchins et al. (1967) reported that metMb accumulation correlated negatively with MRA ($r = -0.44$, $P< 0.01$). Madhavi and Carpenter (1993) suggested many extrinsic factors may affect color stability through MRA, including the manner in which the muscle is processed. Reduction of metMb to myoglobin within the meat system may occur through metMb reductase activity or through nonspecific reduction systems involving intermediates of the glycolytic pathway and the electron transport chain (Saleh and Watts, 1968). Specific metMb reductases have been isolated (Al-Shaibani et al., 1977; Hagler et al., 1979; Levy et al., 1985; Arihara et al., 1989). However, the large number of possible intermediates and substrates in post-rigor muscle, make it difficult to identify essential reactants lost during storage and processing (Ledward, 1985). Probably some blood from neck bones was pressed with the lean. Possibly methemoglobin reductase from red blood cells was present (Kuma, 1981). This enzyme can reduce metmyoglobin and may be a factor in the increased color stability of Baader 1 and Baader 2

lean we observed. Arihara et al. (1989) suggested that metmyoglobin reductase and methemoglobin reductase were the same enzyme. Note that other researchers have reported that MRA was of little consequence in determining meat color (Atkinson and Follet, 1973; O'Keefe and Hood, 1982; Renerre and Labas, 1987). In addition, Ledward et al. (1977) indicated comminution of beef muscle destroyed MRA.

Part of the increased MRA of Baader 1 and Baader 2 lean could be due to pH. Stewart et al. (1965) reported MRA increased from pH 5.1 to 7.1. Ledward (1985) suggested that the rate of metMb reduction increased with increasing pH and the pH dependence was more apparent at pH > 6.0. Baader 1 and Baader 2 lean had pH > 6, significantly higher than hand trim and control. However, correlation between pH and MRA we found was non-significant ($r = 0.37$, $P>0.05$).

Oxidation-reduction potential for hand trim was lower ($P<0.05$) than for all other types of lean or bone marrow (Table 2). We hypothesized that Baader 1 and Baader 2 lean had a lower oxidation-reduction potential and thus a decrease in oxidation of myoglobin; however, that hypothesis was not supported by the observations of our experiments. The lower oxidation-reduction potential of hand trim was likely due to less surface area exposed to oxygen. All other lean types underwent particle reduction of some type and had been stored at −35°C before being powdered.

CONCLUSIONS

MECHANICALLY RECOVERED LEAN from split cervical beef vertebrae may prove beneficial in a coarse ground or an emulsified meat system. The Baader 1 and Baader 2 lean samples had higher ($P<0.05$) MRA, water-holding capacity, pH and total pigment than the control. Such lean sources incorporated into a processed meat formulation could improve color stability and water-holding capacity.

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Reduced Fat, High Moisture Beef Frankfurters as Affected by Chopping Temperature

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ABSTRACT

Six treatment combinations were studied to determine the effects of initial temperature (0, 15, 30°C) and endpoint chopping temperatures (0, 15, 30, 45°C) on texture and stability of reduced fat, high moisture beef frankfurters. Textural properties (raw batter, frankfurter) and purge loss were determined over 8 wk storage. As endpoint chopping temperature increased, batter stability and shear force decreased. In most samples, initial temperature did not affect texture or stability. Endpoint chopping temperatures of ≤ 15°C resulted in most stable batters. Chopping > 15°C lowered product quality.

Key Words: beef-frankfurters, texture, chop temperature, storage stability

INTRODUCTION

THE USDA 40% RULE (Food Safety & Inspection Service, 1988) allows cooked sausages to contain added water and fat that in combination does not exceed 40% of product composition (≤ 30%). This change has led to development of low fat sausages by reducing percent fat and caloric density. However, increasing added water beyond traditional levels alters the sausage batter system. The inability of meat proteins to bind increased amounts of water may explain some excess purge, high yield loss and decreased textural qualities of high moisture batter type sausages (Claus et al., 1990; Hensley and Hand, 1995). Because of such effects, water-binding capacity is the critical issue in production (Rust and Olson, 1988). Therefore, methods are needed to achieve maximum protein-water binding. One method has been to study relationships between temperature of chopping and binding ability in traditional sausages (Helmer and Saffle, 1963; Puolanne et al., 1985). Previous research defined the optimum endpoint chopping temperature to range from 15–23°C (Jones and Mandigo, 1982; Brown and Toledo, 1975). Researchers defined these optimum temperatures for batters with fat contents of ≈ 30% and added water levels < 10%. Commercial reduced fat, high moisture formulations contain much less fat and more added water. Hensley and Hand (1995) studied chopping temperatures in the range 9–15°C and found that low fat, high moisture frankfurters chopped at 12°C were firmer than those from 9 to 15°C. Relationships between initial and endpoint temperatures, and textural and stability parameters of reduced fat, high moisture beef frankfurters have not been reported. Our objectives were to determine the effects of initial and endpoint chopping temperature on texture and stability of reduced fat, high moisture beef frankfurters.

MATERIALS & METHODS

Frankfurter manufacture

Three replications of reduced fat, beef frankfurters were manufactured. Fresh (96 hr postmortem, 4.4°C) 85/15 lean cow trimmings and 50/50 fat beef trimmings, obtained locally, were ground through a 1.27 cm plate (Biro Mfg, Marblehead, OH) and mixed (ribbon-paddle mixer, Leland Mfg., Detroit, MI) 1 min for homogeneity. Samples were removed for determination (AOAC, 1990) of fat (ether extract), moisture (oven drying), and protein (Kjeldahl). Appropriate lean trim, fat trim and water combinations were determined for a 15% fat, 25% added water formulation using least cost formulation (Least Cost Formulator, Virginia Beach, VA). Trimmings were vacuum packaged (M855, Multivac, Kansas City, MO), and frozen (−28.8°C) for about 1 wk prior to production of frankfurters. Raw materials were allowed to thaw at 4.4°C for 36 hr before production. The appropriate amount of lean trimmings and fat trimmings, added water, 2.25% salt, 0.5% sodium tripolyphosphate, 156 ppm sodium nitrite, and 228.6g of spices (A.C. Legg, Birmingham, AL) were weighed and kept separate in sealed bags prior to manufacture. Meat block size was 15.9 kg for all treatments.

Six treatment combinations were studied to determine effects of initial and endpoint chopping temperatures. For the three treatments 0–0, 15–15, and 30–30, the product was chopped (K64 Seydelmann, Robert Reiser, Canton, MA.) for a constant time (8 min) and the initial temperature (0, 15, or 30°C) was maintained with the addition of CO_2 "snow." For treatments 0–15 and 0–30, the product was chopped from an initial temperature (0°C) to the desired endpoint temperature (15 or 30°C). For the final treatment (0–45–15) chopping was from an initial temperature (0°) to 45°C and then immediately chilled with addition of CO_2 snow on the low mix setting to an endpoint temperature of 15°C. This treatment was added to study overchopping as did Helmer and Saffle (1963) in higher fat, lower added-water frankfurters. Prior to manufacturing, all materials (meat, spices, water) were equilibrated to appropriate temperatures in a freezer (0°C), drying chamber (15°C, Alkar, Lodi, WI), or smokehouse (30°C, Alkar, Lodi, WI). Temperature was monitored immediately prior to chopping and addition of other ingredients.

For all treatments, the lean trim, salt and sufficient water for an ionic strength solution of 0.46 were chopped 30 sec at low speed and 2.5 min at high speed. The ionic strength was determined from the first replication in which 1/3 of the required water, the lean fraction and salt were added first. The dry ingredients, remaining water, and fat trim were added and chopped at high speed until the desired endpoint temperature was reached. For those treatments using a constant temperature (initial to endpoint), treatments were chopped an additional 5 min. For the last 30 sec of all treatments chopping was carried out under vacuum. For the 0–15, 0–30 and 0–45–15 treatments chopping times were averages of 7, 14 and 45 min, respectively.

Each batter was stuffed into 32 mm cellulose casings (EZ Peel Nojax, Viskase, Chicago, IL) and heat-processed (smokehouse, Alkar, Lodi, WI) (see smokehouse schedule Table 1). Cooked weights were recorded after frankfurters had been hand showered with cold tap water to an internal temperature of 38°C. Yields were determined by dividing the cooked weight by the raw-stuffed weight and multiplying × 100. Frankfurters were allowed to cool 12 hr at 4.4°C before being peeled (Ranger Apollo, Townsend Engineering, Des Moines, IA). Links were vacuum packaged (M855, Multivac, Kansas City, MO), 5 links/package and stored at 4.4°C for purge analysis.

Table 1—Thermal processing schedule for reduced fat, beef frankfurters

Method	Dry bulb (°C)	Wet bulb (°C)	Time (min)
Cook	62.8	—	20
Smoke and cook	68.3	—	30
Smoke and cook	73.8	56.7	35
Smoke and cook	85.0	68.8	To internal endpoint temp (71°C)
Cold shower	—	—	To internal endpoint temp (38°C)

Batter properties

Batter stability was determined by the method of Townsend et al. (1968). Raw batter (34g) was placed into a plastic tube, covered with

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Table 2—Yield and proximate composition of reduced fat, high moisture beef frankfurters

Treatment[a] °C, min	Protein (%)	Moisture (%)	Fat (%)	AW (%)[b] (calculated)	Yield (%)[c]
0–0,8	13.0^{d} (0.01)	69.6^{de} (0.3)	13.5^{g} (0.02)	17.6^{d} (0.1)	88.4^{d} (0.6)
0–15,7	13.1^{d} (0.02)	69.0^{ef} (0.1)	14.1^{fg} (0.02)	16.6^{de} (0.2)	87.2^{d} (0.6)
0–30,14	13.4^{d} (0.01)	69.2^{def} (0.2)	14.8^{e} (0.02)	15.6^{de} (0.0)	87.6^{d} (0.6)
0–45–15,45	13.9^{d} (0.02)	64.4^{g} (0.6)	17.0^{d} (0.2)	8.8^{f} (0.1)	86.7^{d} (0.4)
15–15,8	13.5^{d} (0.06)	68.7^{f} (0.1)	14.1^{fg} (0.02)	14.8^{e} (0.1)	85.7^{d} (0.3)
30–30,8	13.3^{d} (0.06)	69.8^{d} (0.3)	13.6^{fg} (0.09)	15.3^{de} (0.2)	88.2^{d} (0.3)

[a] Initial and endpoint temperatures in °C, Chopping times in min.
[b] Added water=% moisture −(4 × % protein).
[c] Yield=cooked weight divided by raw weight × 100.
[defg] Means within a column followed by a different superscript are different ($P < 0.05$). Standard errors are in parenthesis.

Table 3—Influence of chopping temperature on batter stability

Treatment[a]	Batter stability: Total loss (%)	Batter stability: Purge (%)
0–0,8	2.7^{d} (1.2)	2.5^{b} (0.1)
0–15,7	2.3^{d} (1.0)	2.0^{c} (0.1)
0–30,14	4.9^{c} (1.3)	2.0^{c} (0.1)
0–45–15,45	8.4^{b} (0.6)	1.2^{d} (0.1)
15–15,8	1.7^{d} (1.5)	2.0^{c} (0.1)
30–30,8	4.9^{c} (1.6)	2.7^{b} (0.2)

[a] Initial and endpoint temperatures in °C and chopping times in min.
[bcd] Means within columns followed by different superscripts are different ($P < 0.05$). Standard errors in parenthesis.

plastic film (Parafilm, American National Can, Greenwich, CT) and heated in a 48.8°C water bath (Model BKS-350, Gallencamp and Co., Sussex, England). After 30 min the temperature was increased intermittently until the internal temperature of the batter reached 68.8°C. One tube in each batch had a thermometer. Released fluids were decanted into 15 mL tubes and centrifuged (Model J-6M, Beckman Instrument, Palo Alto, CA) for 1 min at 2.68 × g. After chilling (4.4°C, 12 hr), the total volume of fluid released was recorded and expressed as percentage of sample weight.

Textural measurements

Raw batter extrusion was performed (triplicate) immediately after stuffing according to a modification of the Voisey and Larmond (1971) method. Values were determined by packing a cell attached to an Instron Universal Testing Machine (Model 4500, Instron Corp. MA), with 300 g batter and compressing at a cross head speed of 100 mm/min to a distance of 0.5 cm from the base. A 10 kN load cell was used. Peak force and total energy were used to determine extrusion values.

Frankfurter textural measurements were made on samples that had been cooked, packaged and refrigerated (4.4°C) 1 wk prior to testing. Compression values (quadruplicate) were determined by axially compressing 65% of the height (Voisey, 1977) of the short axis of a 3 cm sample (4.4°C) cut from the center of the frankfurter. The sample was placed between plates attached to an Instron Universal Testing Machine set at a crosshead speed of 500 mm/min with a 1 kN load cell and compressed twice. Values obtained were hardness and cohesiveness (Singh et al., 1985).

Kramer shear values (quadruplicate) were obtained by shearing the short axis of a 4 cm frankfurter sample (4.4°C) cut from the center of the frankfurter. The sample was placed perpendicular to the blades of a Kramer shear attachment and the Instron had a crosshead speed of 100 mm/min with a load cell of 10 kN. Peak force and area under the curve (energy) were used to determine shear measurements (Singh et al., 1985).

Compositional and purge analyses

Raw materials and finished product proximate composition (moisture, fat, protein) were analyzed according to AOAC (1990) procedures (drying oven, ether extract, Kjeldhal). Starting 1 wk after production, purge was measured once a week on stored products for 8 wk. Two random packages from each treatment were weighed, opened, placed in a plastic funnel and drained of purge (5 min) and reweighed. The package film was dried at 100°C for 2 hr to remove residual purge from the corners. Purge loss was recorded as a percentage of total frankfurter weight after draining divided by initial packaged weight.

Statistical analysis

Statistical analysis was performed using the Statistical Analysis System (SAS, 1985). Main treatments (6) with 3 replications from this randomized complete block design were analyzed by 1-way analysis of variance. Treatment means were separated using least significant difference procedures (Steel and Torrie, 1980).

RESULTS & DISCUSSION

Smokehouse yield and composition

Smokehouse yields (Table 2) were not different ($P > 0.05$) for any treatments Hensley and Hand (1994) reported similar results in frankfurters of varying fat and added water levels. As formulated, percent protein for all treatments was not different ($P > 0.05$). Treatments 0–0, 0–15, 0–30 and 30–30 had the highest ($P < 0.05$) percentages of moisture and conversely the lowest percentage fat (Table 2). Since there were no differences in smokehouse yields, lower moisture values than the targets may have been due to loss of moisture during chilling since samples for proximate composition analyses were removed after chilling. The 0–45–15 treatment was higher ($P < 0.05$) in fat and lower in moisture than all other treatments.

Batter stability

Batter stability (Table 3) data indicated that the treatment with the greatest peak temperature (0–45–15) had more total loss, and was thus less stable, than all other treatments. Helmer and Saffle (1963) reported complete batter breakdown when chopping at > 28°C. The two treatments that had endpoint chopping temperatures of 30°C (0–30 and 30–30) showed similar ($P > 0.05$) total losses, but were higher ($P < 0.05$) than treatments 0–0, 0–15 or 15–15. Jones and Mandigo (1982) also reported a trend for traditional fat frankfurters chopped at >22°C to have higher total batter loss than treatments chopped to endpoint temperature 16°C. Treatments 0–0, 0–15 and 15–15 were not different ($P > 0.05$) for total loss and had the lowest ($P < 0.05$) losses of all treatments. Similar results had been reported by Puolanne et al. (1985) in which water binding values remained constant between 12° and 20°C and then decreased at > 20°C.

Textural properties

Textural analysis of raw frankfurter batter and cooked frankfurters (Table 4) showed higher values for both measurements, indicating a firmer, more stable product. Treatment 0–0 had the highest ($P < 0.05$) values for both raw frankfurter batter peak force and energy. The 0–15 and 15–15 treatments were not different ($P > 0.05$) and were higher ($P < 0.05$) than treatments chopped at > 15°C (0–30, 30–30, and 0–45–15). The 0–45–15 treatment had the lowest ($P < 0.05$) value for peak force and energy. These raw batter data corresponded to batter stability data, in which the 0–0, 0–15, and 15–15 treatments were not different ($P > 0.05$) from each other and were higher ($P < 0.05$) than those for treatments chopped at > 15°C. With exception

Table 4—Influence of chopping temperature on textural measurements of raw frankfurter batter and cooked frankfurters

	Raw batter		Cooked frankfurters		
	Extrusion		Compression	Kramer shear	
Treatment[a]	Energy (J)	Force (N)	Hardness (N)	Energy (J)	Force (N)
0–0,8	1.4[b] (0.3)	0.031[b] (0.006)	0.23[c] (0.05)	2.5[b] (0.9)	0.40[c] (0.17)
0–15,7	1.3[c] (0.2)	0.026[c] (0.004)	0.20[c] (0.03)	2.6[b] (0.7)	0.45[b] (0.13)
0–30,14	1.0[d] (0.2)	0.020[d] (0.002)	0.24[c] (0.06)	2.0[c] (0.8)	0.33[d] (0.15)
0–45–15,45	0.1[f] (0.2)	0.009[e] (0.004)	0.33[b] (0.07)	1.4[d] (0.3)	0.20[f] (0.05)
15–15,8	1.3[c] (0.1)	0.027[c] (0.002)	0.22[c] (0.09)	2.6[b] (1.1)	0.38[c] (0.16)
30–30,8	0.9[e] (0.2)	0.022[d] (0.005)	0.21[c] (0.04)	1.8[c] (0.4)	0.29[e] (0.08)

[a] Initial and endpoint temperatures in °C, chopping times were in min.
[bcdef] Means within columns followed by different superscripts are different ($P < 0.05$). Standard errors in parenthesis.

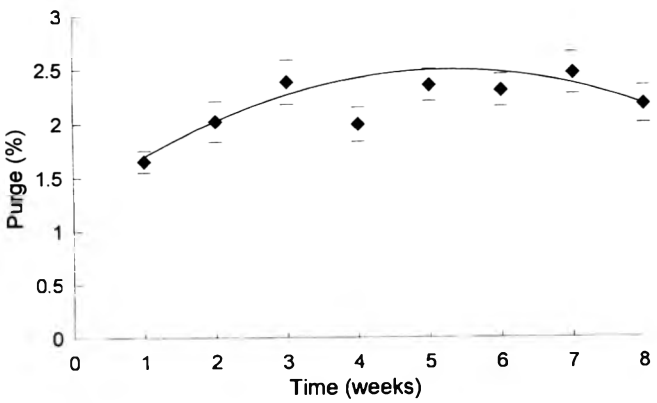

Fig. 1—Frankfurter purge as influenced by storage period. Regression line, means (♦) and standard errors (–).

of the 0–45–15 treatment, these extrusion values tended to follow the endpoint chopping temperatures.

Compression measurements were used to determine cohesiveness and hardness. Treatments were not different ($P > 0.05$) for cohesiveness values (mean=1.00, SE 0.18). Claus et al. (1990) reported similar results for low fat, high moisture bologna. Analysis of hardness (Table 4) showed that the 0–45–15 treatment required the greatest force to compress the sample to 65% of its original height. This was probably a reflection of lower moisture values. Brady and Hunecke (1985) found a similar negative correlation between hardness and moisture values. All other treatments were not different ($P > 0.05$) from each other.

Kramer energy shear values indicated that chopping to endpoints > 15°C (0–30, 30–30 and 0–45–15) required less ($P < 0.05$) energy to shear than chopping to peak temperatures of ≤ 15°C. Treatments with endpoint chopping temperature of 30°C (0–30 and 30–30) were not different ($P > 0.05$) from each other and required more energy than the 0–45–15 treatment. In addition, 0–0, 0–15 and 15–15 were not different ($P < 0.05$) from each other. As expected from raw batter analysis, the 0–45–15 treatment was softest. This trend for reduction in shear force as peak chopping temperature increased had been reported by Brown and Ledward (1987) in a study on effects of temperature of comminution on stability and eating quality of 'English' sausages. That study showed toughness of sausages decreased ($P < 0.001$) with increased temperature of comminution.

Numeric values for Kramer shear force (Table 4) decreased as endpoint chopping temperature increased with exception of 0–15. The 0–15 treatment showed the highest value ($P < 0.05$) while treatment 0–45–15 had the lowest value ($P < 0.01$). Loss of bind for the 0–45–15 treatment could be related to prolonged and excessive mechanical action. Webb et al. (1975) stated that a great degree of protein denaturation occurs under such conditions resulting in less protein-water binding.

Purge

The regression line was fitted (Fig. 1) over all treatments, means and standard errors for the percentage purge for the 8 wk period. Purge increased in a quadratic or curvilinear fashion with a maximum at about week 5 and then decreased slightly. These findings were different from those of Hensley and Hand (1994) who found purge increased linearly over an 8 wk frankfurter storage. However, mean values for similarly formulated products were in the same range. Although statistical differences and a curvilinear relationship are shown, meaningful conclusions are not clear due to the small magnitude in the range of means (1.6 to 2.5%).

Total purge (Table 3) was the greatest for treatments 0–0 and 30–30. These findings confirmed those of Harbitz and Egelandsdal (1983) who found reduced binding ability for frankfurter batters chopped at < 0°C and Helmer and Saffle (1963) who noted complete batter breakdown of sausage batters chopped at > 28°C. The 0–45–15 treatment had the least purge; however, that treatment also had significantly less moisture than all other treatments.

CONCLUSIONS

As peak chopping temperature increased, batter stability and shear force decreased. In most cases, initial raw material temperature did not affect quality parameters. Treatments with peak temperatures of 15°C and below (0–0, 0–15 and 15–15) were most stable. As with traditional frankfurters, chopping at > 15°C was detrimental to quality in reduced fat high moisture beef frankfurters.

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Cooking Treatment, Mixing Time, and Mixing Temperature Affect Pepperoni Cupping

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ABSTRACT

Pepperoni was manufactured using three mixing temperatures (−5, 0, 5°C), three mixing times (2, 8, 14 min) and two cooking treatments (uncooked or cooked to 60°C) to evaluate effects on endpoint textural parameters and cupping of pepperoni. Covariance analysis resulted in higher cohesiveness values and lower cupping scores ($P<0.05$) for the −5°C treatments than for the 0 or 5°C mixed treatments. Cooked pepperoni had less diameter shrink ($P<0.05$) and higher cupping scores ($P<0.05$) than uncooked treatments. Path analysis (standard partial regression coefficients) showed that diameter and cohesiveness had the greatest direct effects on cupping.

Key Words: pepperoni, cupping, mix time, cook time, thermal effects

INTRODUCTION

DRY SAUSAGES, such as pepperoni, are dried from 12 to 60 days (Hoogenkamp, 1989; Terrell et al., 1977) to develop characteristic textures and flavors (Everson et al., 1970), provide extended shelf-life (Townsend et al., 1980), and control trichinae to meet regulations (Food Safety & Inspection Service, 1990). However, drying results in textural changes, some of which may be detrimental to consumer acceptability of the product. Over the drying period, decreases in moisture (Wardlaw et al., 1973), sausage diameter (Keller et al., 1974) along with increased shear values (Acton and Keller, 1974; Keller et al., 1974; Wardlaw et al., 1973) have been reported.

One detrimental textural property unique to pepperoni is described as cupping (Hoogenkamp, 1989; Newkirk et al., 1993). This is the curling of pepperoni slices when they are cooked on a pizza and may be objectionable to consumers. The textural properties of some meat products have been shown to change due to increased mixing (Booren et al., 1981 a, b, c). Increased temperature affects protein extraction (Hamm, 1960; Gadea de Lopez and Hand, 1993) and increased mixing temperatures affect properties of restructured steaks (Popenhagen and Mandigo, 1978). The heating of pepperoni sausages caused textural changes (Palumbo et al., 1976a). Such manufacturing effects have been studied in many types of meat systems, but no research has been published on the effects of such treatments on textural parameters in dry sausage such as pepperoni. Our objective was to examine the manufacturing condition of cooking treatment, mixing temperature, and mixing time on cupping of pepperoni.

MATERIALS & METHODS

Pepperoni preparation

Frozen boneless cow trim (−29 ± 2°C, 1-3 mo postmortem) was obtained from the Oklahoma State University meat laboratory and fresh boneless pork shoulders (2 ± 2°C, 48-96 h postmortem) were purchased from local suppliers. Beef trim was thawed at 2 ± 2°C for 24 hr. Beef and pork portions were ground (Biro Mfg., Marblehead, OH) separately through a 12.7 mm plate, mixed (ribbon-paddle, Model L100DA, Detroit, Mfg., Detroit) 1 min for homogeneity and sampled for proximate analysis. Portions were then stored 48 hr at 2 ± 2°C. Nine 15-kg batches were prepared at 1:1 beef to pork ratio, both consisting of 22% fat. Meat batches were tempered overnight to 5°C before pepperoni manufacture.

Pepperoni batches were mixed in a twin-shaft paddle mixer (U-Mec model 320, Hayward, CA) with paddles rotating at 25 rpm. Batches were mixed at either −5, 0 or 5°C for 2, 8 or 14 min. Prior to mixing, the meat batch temperature was equilibrated to the appropriate mixing temperature by addition of CO_2 "snow." Temperature throughout the manufacture was maintained at ± 1°C with the addition of CO_2 snow. The spice mixture (1.48%, A.C. Legg, Birmingham, AL) and dextrose (0.5%) was added during the first minute of mixing, followed by $NaNO_2$ (156 ppm), salt (2.15%) and antioxidant mixture (0.006%, Tenox 6, Eastman Kodak). The starter culture (0.5%, Diversitech HP, Diversitech, Gainesville, FL) was added last according to manufacturer directions.

After mixing, pepperoni mixtures were reground through a 4.7 mm plate, then vacuum-stuffed (Vemag Robot 500, Robert Reiser, Canton, MA) into 45 mm fibrous cellulose casings (IR-60, Viskase, Chicago, IL) resulting in 0.46 kg sticks. During stuffing, samples were taken for pH determination and proximate analysis. Three replications of experimental batches were manufactured in different weeks using different lots of beef and pork.

Pepperoni fermentation and processing

Pepperoni was placed into a computer controlled one-truck smokehouse (Alkar; Lodi, WI) and fermented 10 hr at 38°C and 85% relative humidity. After fermentation, each treatment combination (a mixing time and mixing temperature) was divided into two cooking treatments: (1) fermented-uncooked and (2) fermented-cooked. The uncooked sticks were then transferred to a one-truck drying chamber (Alkar, Lodi, WI) with initial dry-bulb setting of 15.6°C and wet-bulb setting of 13.3°C and an airspeed of 12 m/min. During drying, the wet-bulb setting was decreased slightly until moisture:protein ratios reached 1.6:1. After fermentation, cooked treatment sticks were thermally processed in a smokehouse controlled by a step program (DDC, Alkar; Lodi, WI) to an endpoint temperature of 50°C (Table 1). After thermal processing, cooked treatment sticks were also transferred to the drying chamber.

Sausages were dried to reach an average endpoint moisture:protein ratio of ≈1.6:1. Previous trials using the same system indicated drying times of 11 days were required for fermented-cooked treatments and 13 days for fermented-uncooked treatments.

pH

Samples were checked for pH after fermentation to assure that the pepperoni achieved a pH ≤ 5.0. pH was determined after drying using a modified method described by Keller et al. (1974). Modifications included blending the 10-g samples of meat in 100 mL distilled water with a Polytron Laboratory Blender (Kinematica, Luzerne, Switzerland) for 60 sec.

Table 1—Smokehouse schedule for pepperoni cooking

Method	Dry bulb (°C)	Wet bulb (°C)	Time
Smoke and cook	49	45	1 hr
Smoke and cook	55	50	1 hr
Smoke and cook	65	60	Until internal temp ≥ 60°C
Hot shower	—	—	3 min
Cold shower	—	—	7 min

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Table 2—Means and standard errors for endpoint moisture:protein ratios

Treatment		Moisture:protein ratios	
Mixing temp (°C)	Mixing time (min)	Cooking treatment: Cooked	Cooking treatment: Uncooked
−5	2	1.59[a] (0.21)	1.48[a] (0.15)
	8	1.57[a] (0.21)	1.32[a] (0.15)
	14	1.44[a] (0.21)	1.32[a] (0.12)
0	2	1.80[a] (0.12)	1.35[a] (0.21)
	8	1.51[b] (0.21)	1.51[a] (0.21)
	14	1.52[b] (0.21)	1.33[a] (0.21)
5	2	1.40[b] (0.12)	1.48[a] (0.21)
	8	1.79[a] (0.12)	1.44[a] (0.15)
	14	1.76[a] (0.11)	1.59[a] (0.15)

[ab] Means within the same column with unlike superscripts are different ($P<0.05$).

Table 3—Diameter change and cupping evaluations in cooked and uncooked pepperoni[a]

	Treatment	Measurement
%Diameter	cooked	88.38 (0.39)[b]
	uncooked	86.12 (0.48)[c]
Evaluator scores	cooked	3.21 (0.10)[b]
	uncooked	2.74 (0.08)[c]
Height measurement	cooked	9.84 (0.23)[b]
	uncooked	9.20 (0.19)[c]

[a] Parameters adjusted by covariance to mean moisture:protein ratio of 1.53:1.
[bc] Means for the same parameter with unlike superscripts are different ($P<0.05$).

Yield and diameter changes

Six sausage sticks/treatment were weighed immediately after stuffing. The same six sticks/treatment were reweighed and measured for stick diameter (midpoint longitudinally) after drying.

Texture profile

Hardness and cohesiveness measurements were conducted on an Instron Universal Testing Machine (Model 4500, Instron Corp., MA) using a modified method (Voisey, 1977). Modifications included axially compressing 62.5% of the height of the short axis of a 3-cm long sample cut from the center of the pepperoni stick for 2 cycles. Compression speed was 50 mm/min with a 10-kg load cell. No dwell time at the bottom of the compression stroke was employed. The height of the first compression peak force represented the hardness measurement and the ratio of peak 2 to peak 1 force was used as the cohesiveness measure. Pepperoni stick samples (in duplicate) were removed for analysis after drying. Samples were held overnight at 4°C in plastic bags, then allowed to equilibrate to room temperature (≈23°C) before analysis.

Proximate analysis

Moisture (oven drying), fat (ether extraction) and protein (Kjeldahl nitrogen) were performed according to AOAC (1985) procedures. Raw materials and formulated batches were analyzed for formulation purposes and pepperoni sticks (duplicate) were analyzed at the end of drying.

Cupping evaluation

Ten pepperoni slices were arranged in three rows on 25.4 cm frozen cheese pizzas (Jeno's, Kansas City, MO). Slices were placed on pizzas in a specific pattern so as to assign a location number for each slice on each pizza for later analysis. Three pizzas per replication were prepared for each of the 18 mixing time-mixing temperature-cook treatment combinations (162 total pizzas). Pizzas were then cooked in a conveyer-fed impingement oven (Model 1022, Lincoln, Ft. Wayne, IN) for 3.5 min at 260°C. Pizzas for each separate replication were cooked on different days. Pizzas were evaluated for cupping by two methods: (1) subjective—a trained panel evaluation, and (2) objective—cupping height.

Panelist evaluation

Ten panelists were trained in two 30-min sessions to evaluate cupping of pepperoni slices on a 4-point scale (1 = flat, 2 = wrinkled, 3 = moderately cupped, 4 = fully cupped). Training was performed using sample pizzas which displayed the various ranges of pepperoni cupping. Panelists were selected based on repeatability on identical samples. Panelists were then presented pizzas in a random order and assigned cupping scores to each slice of pepperoni (1–10) on each experimental pizza.

Cupping height

Pepperoni slices from locations 1, 5, 6 and 10 were removed from each pizza and measured for cupping height (mm) by two evaluators. These locations represented two outside edges (1, 10) and two interior slices (5, 6). Slices were removed from the pizzas, excess cheese removed and placed upside down on a Plexiglas® board. A height measurement from the highest point of the pepperoni slice to the bottom of the Plexiglas® was taken with a micrometer. The height of the Plexiglas® was then subtracted.

Statistical analysis

Statistical analysis was performed using the Statistical Analysis System (SAS Institute, Inc., 1985). The 2×3×3 split plot design with randomized complete replications (n=3) was analyzed by one-way analysis of variance and where appropriate, means were differentiated by least squares means. In order to remove any variation due to differences in moisture:protein ratios, covariance using the mean endpoint moisture:protein ratio as the covariant was employed to analyze differences between parameters. Additionally, path coefficients (standard partial regression coefficients) were computed to assess individual and joint contributions of various characteristics that influence cupping (Wright, 1934; May et al., 1992).

RESULTS & DISCUSSION

ANALYSIS OF FINAL MOISTURE:PROTEIN RATIOS (Table 2) showed a three-way interaction ($P<0.05$) between cooking treatment, mixing time and mixing temperature. There was no difference ($P>0.05$) between treatments for any uncooked batches. In general, the uncooked pepperoni displayed lower moisture:protein ratios than cooked treatments, which could be attributed to an additional 2 days of drying. The −5°C mixing temperature, cooked products were not different ($P>0.05$) among mixing times. For both the 0 and 5°C mixing temperature, cooking treatments, the 2 min mixing time gave different results ($P<0.05$) from the other two mixing time treatments. However, the 2 min mixing time had a higher moisture:protein ratio than the mixing times samples for the 0°C treatment, and a lower ratio for the 5°C treatment than the other mixing treatments. To more appropriately compare and analyze treatments, covariance, using the mean endpoint moisture:protein ratio (1.53:1) as covariant, was used to analyze data.

Covariance analysis showed no difference ($P>0.05$) between treatments for the parameters of pH (mean = 4.6, SE 0.01), yield (mean = 66.1%, SE 0.71) or texture profile hardness (mean = 0.53 N, SE 0.81). Our pH values agreed with those found by Townsend et al. (1980), but were slightly lower than those reported in the range 4.7–4.9 by Palumbo et al. (1976b) in a pilot plant process for pepperoni. Mean yields were slightly lower than those reported by Keller et al. (1974) of ≈77% at 10 days drying and 69% at 15 days drying, but were slightly higher than the ≈60% yield after 14 days drying predicted by Palumbo et al. (1977). The differences were probably related to the relationship of yield and moisture:protein ratios.

Cooked pepperoni maintained a higher ($P<0.05$) percent diameter than did uncooked pepperoni (Table 3). The higher mean percent diameter for cooked treatments could be attributed to firmness development of the sausage due to the 60°C treatment. Palumbo et al. (1976a) reported that sausages fermented and heated to 60°C, developed a firm texture upon drying, while nonfermented, nonheated sausage developed softer texture (cursory observation). However, no objective data on texture was presented. Palumbo et al. (1977) stated that heating increased the homogeneity of the mixture as melted fat was redistributed in the product. This redistribution resulted in higher percent yields for heated compared with nonheated pepperoni after 42

days drying. Our results showed no difference in percent yield between cooked and uncooked pepperoni at the same moisture: protein ratio, but the uncooked sausage took an additional 2 days of drying to reach the required average moisture:protein ratio of 1.6:1.

Several researchers have shown that sausages became firmer as they dried (Townsend et al., 1980; Lu and Townsend, 1973; Keller et al., 1974). Shear values have been evaluated but the texture parameter of cohesiveness has not been reported for dry sausage. Shear values measure the rupture force of particles in the sausage, but cyclic compression measures the binding strength between particles. Cyclic compression was used because of the potential of the treatments to contribute more to particle binding than to particle shear force. There was no difference ($P>0.05$) between cooked and uncooked pepperoni, but differences ($P<0.05$) were found for different mixing temperatures. The −5°C mixed treatment samples (mean = 0.37, SE 0.009) had higher ($P<0.05$) cohesiveness values than the 0° (mean = 0.34, SE 0.010) or 5°C (mean = 0.33, SE 0.009) treatment samples, which were not different. Cohesiveness values were calculated by dividing the force at height of the second peak of the cyclic compression cycle by the force at height of the first peak (Voisey, 1977). Since there was no difference in hardness which is related to height of the first peak (Bourne et al., 1966), differences in cohesiveness occurring in the products appeared in the second deformation curve. Possibly, the −5°C treatments displayed a higher degree of elasticity between compression cycles, giving higher cohesiveness values. Visual observation showed less fracturing of the −5°C product upon the first compression cycle, which would indicate more elasticity in the product.

Table 4—Measurements for cupping evaluation and cupping height measurements for pepperoni mixed at different temperatures or subjected to different cooking treatments[a]

	Temp (°C)	Measurement
Evaluator score	−5	2.51 (0.10)[c]
	0	3.04 (0.12)[b]
	5	3.37 (0.10)[b]
Height measurement (mm)	−5	8.63 (0.25)[c]
	0	9.59 (0.30)[b]
	5	10.34 (0.25)[b]

[a] Parameters adjusted by covariance to a mean moisture:protein ratio of 1.53:1.
[bc] Means for the same parameter with unlike superscripts are different ($P<0.05$).

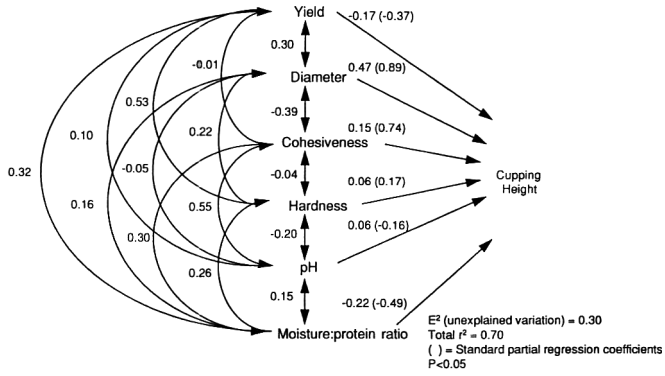

Fig. 1—Path coefficient diagram for selected variables of cupping height measurement.

Cupping evaluation

Panelist scores and cupping height measurements (Table 3) based on subjective and objective evaluations were correlated with a correlation coefficient of 0.79 ($P<0.05$). Both types measurements indicated lower cupping values for uncooked pepperoni ($P<0.05$). Hamm and Deatherage (1960) showed that heating of meat to 60°C at pH 5.0 rendered many structural proteins insoluble. Therefore, while heating decreased drying time, it also created textural changes which caused the cooked pepperoni to cup more extensively.

Panelist and cupping measurements were also influenced by temperature (Table 4). Panelist and cupping height measurements showed the −5°C treatment samples had the least amount of cupping ($P<0.05$). There was little difference ($P>0.05$) between the 0 and 5°C treatments. These higher mixing temperatures tended to extract more protein in the sausages, and thus dried at a slower rate, as evidenced by higher endpoint moisture: protein ratios. However, moisture:protein ratio was held constant for covariance analysis. Popenhagen and Mandigo (1978) found that as temperatures for flaked and formed steak products were increased from −5.6°C to 0.6°C, adhesion between meat particles increased. This increase could also lead to a greater amount of retained moisture in the 0 and 5°C mixed treatments due to increased water binding ability. Hoogenkamp (1989) stated that cupping was caused by a differential in moisture between the outer edge of the slice and the inner edge. Therefore, increased mixing temperatures from −5°C to 0 or 5°C could lead to increased protein extraction and thus a more steep moisture gradient in pepperoni. During drying, moisture would be most easily lost from the outer edges of products, leaving the interior with more moisture.

The main effects of cooking time, mixing temperature and mixing time accounted for 70% of the variation in cupping height. Remaining variation was analyzed to determine which parameters could possibly serve as indicators of cupping. Relationships between various measurements and cupping height were used to construct a path analysis for cupping height (Fig. 1). Path analysis was also conducted for evaluator scores with similar results, but only the cupping height path is presented here. The path coefficients (standard partial regression coefficients) are shown in parentheses on the straight single-headed arrows. Squaring a path coefficient gives the percentage of variation in cupping accounted for by the direct effect of that parameter, holding other variables constant. For example, the direct effect of diameter on cupping height accounted for 79% of the variation in cupping height ($0.89^2 = 0.79$). For cupping height, the paths in the diagram accounted for 70% of the variation in cupping height. Diameter (79%) and cohesiveness (55%) had the largest direct effect on height measurement.

Additionally, each variable had an indirect effect through its correlation with other variables. These effects, again holding other variables constant, could be found by multiplying the correlation between variables and the path coefficient between the second variable and the cupping height. For example, the indirect effect of diameter through cohesiveness on cupping height was $-0.39 \times 0.74 = -0.29$. All additional numbers located on the arrows (single- and double-headed) represent simple correlations between those parameters. Since diameter and cohesiveness had the greatest direct effect on cupping, the indirect effects with the largest impact on cupping height were those routed through either diameter or cohesiveness. The order of importance (absolute value) for indirect paths through diameter was cohesiveness (−0.35), yield (0.27), hardness (0.20), moisture: protein ratio (0.14) and pH (−0.05). The order of importance for indirect paths through cohesiveness was pH (0.41), diameter (−0.29), moisture:protein ratio (0.22), hardness (−0.03) and yield (−0.01).

Changes in diameter and cohesiveness brought about by drying seemed to affect the degree of cupping. When the negative indirect path of cohesiveness through diameter was considered (−0.35), it appeared that a decrease in a combination of those parameters increased the incidence of cupping. This tended to agree with the covariance analysis for cohesiveness, which showed that as cohesiveness decreased ($P<0.05$) between the −5°C and the 0 and 5°C treatments, cupping height tended to increase.

CONCLUSIONS

UNCOOKED PEPPERONI tended to cup less than cooked pepperoni, and that mixed at −5°C also tended to cup less than that from other warmer mixing temperatures. Path analysis showed diameter and cohesiveness were the best predictors of cupping. Therefore, processors may be able to use changes in diameter and cohesiveness of pepperoni as it dries to predict cupping of the finished product.

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Quality Evaluation of Chile-flavored, Jerky-type Extruded Products from Meat and Potato Flour

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ABSTRACT

Extruded, jerky-type products prepared from potato flour combined with either partially defatted chopped beef (PDCB), mechanically separated chicken (MSC) or chicken thigh meat (C) and flavored with three levels of chile powder were evaluated by instruments and sensory methods. Meaty aroma was more intense than potato or chile aromas in all samples. MSC product tended to have the darkest color. Shear strength, tensile strength and sensory analyses indicated chile powder may be a binding agent, resulting in harder texture, particularly in C products. Meaty and black pepper were dominant flavor characteristics detected by sensory panelists. Overall, PDCB product containing 0.5% chile was rated most desirable.

Key Words: jerky, chicken, beef, extruded, chile

INTRODUCTION

SNACK FOODS represent a high value segment of the food industry in the U.S. ($12 billion, Dillon, 1990). Convenient, small portions to satisfy short-term hunger, snacks range from chips, pretzels and crackers to fruit rolls, cookies and granola bars (Tettweiler, 1991). Meat snacks, however, represented only 5% of the total snack food revenues in 1990 (Dillon, 1990). In response to demands for more nutritious, reduced fat, sodium and calorie products, as well as to increase the meat industry share of the snack food markets, development is growing for meat snack products from extrusion of meats and their by-products (Shaw. 1990).

Worldwide, extruded products may soon replace nuts as the third most popular snack food category, after chips and sticks (Tettweiler, 1991). Extrusion involves forcing a dough-like mass through shaped openings under controlled conditions of temperature, pressure and rate of flow (Dziezak, 1989). Cereal-based products have been the most common foods produced by extrusion, but others range from confectionary items to animal foods (Dziezak, 1989).

Meat jerky has long been a major source for sustenance. It was used as "charqui" by Indians of South America, "pemmican" by North American Indians in the late 15th century and as the "biltong" of South Africa (Sharp, 1953; Tannahill, 1973; Romans and Ziegler, 1974). Jerky is a high protein, light-weight, shelf-stable meat snack popular with campers, hikers and many general consumers.

Traditionally, jerky has been made from thinly sliced whole muscles (of large bovine animals) which have been brined and dried. Miller et al. (1988) prepared jerky from lesser used organ meats such as beef heart and tongue. Ray (1994) and colleagues used a single-screw, high temperature, short time extruder to prepare jerky-type products from pre-gelatinized potato flour combined with either partially defatted chopped beef, mechanically separated chicken or chicken thigh meat. Nutritional analysis indicated the products were lower in fat and cholesterol than traditional jerky products.

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Our objective was to evaluate the jerky products reported by Ray (1994) using both sensory evaluation and instrumental measurements of color and texture.

MATERIALS & METHODS

Material acquisition

Potato flour was obtained from Napariel® Corp. (Blackfoot, ID). Frozen chips of partially defatted chopped beef (PDCB; Beef Products Inc., Austin, TX) and frozen mechanically separated chicken (MSC; O.K. Foods Inc., Fort Smith, AR) were expressed and placed in a −23°C freezer immediately upon arrival. Samples were used within 45 days of shipping. Fresh hindquarters of chicken (C) were obtained from a local supermarket, deboned. trimmed of external/internal fat and skin and frozen (−23°C).

The frozen PDCB, MSC, and C were cut into 5 cm × 7 cm × 5 cm strips with a Biro® band saw (Model 44, Biro Manufacturing Co., Marblehead, OH). The frozen strips were ground through a 0.38 cm plate using a Hobart® grinder (Model 4056, Hobart Manufacturing Corp., Troy, OH) and stored (−23°C) until immediately before blend preparation.

Blend preparation

In preparation of each blend for jerky-type products, powdered spices/seasonings/flavorings were mixed with potato flour using a Kitchen Aid® mixer (Model KSM5PSGR, Kitchen Aid, Inc., St. Joseph, MI) for 2 min. The seasoned potato flour was then mixed with either PDCB, MSC or C using a Waring® 7-speed Vortex Blendor (Model 153L17, Waring Product Division, New Haven, CT) for 1 min. The jerky-type blends were seasoned with a combination of eight spices/seasonings/flavors added at the same level in all preparations. In addition to the spice blend, black pepper was added to each batch at 0.7 g/200g blend. Chile seasoning prepared from ground, dried chile peppers (AJI; 9,000 Scoville units) was added to batches of each meat type at each of the following levels: (1) 1g AJI/200 g blend (0.5%); (2) 2g AJI/200g blend (1.0%); and (3) 3g AJI/200g blend (1.5%). The nine meat type/chile level blends were then stored (4°C) for 24 hr prior to extrusion processing.

Extrusion processing

A single-screw, laboratory extruder (Model 2003, C.W. Brabender Instruments, Inc., South Hackensack, NJ) was used. The extruder was fitted with a barrel of 1.91 cm diameter and 20:1 length/diameter (L × D). An alloy (4140), hard chrome plated, single flight screw, with uniform pitch and a 3:1 compression screw were used. The extrudate exited the extruder through a ribbon die (5 cm × 2 mm) to form a strip of product. The extruder was equipped with temperature controls for feed, compression, and metering sections of the barrel, torque, temperature and pressure monitors, a variable speed motor (0-250 rpm), and a Dynisco® pressure and temperature transducer (Model TPT-432A, Dynisco®, Sharon, MA).

Preliminary trials were conducted to determine extrusion conditions. For each extrusion run, care was taken to ensure that the screw flight at the feeding part was kept full throughout the process. The jerky-type products were extruded under the following conditions: 160 rpm screw speed, 40–50°C for the feeder section and 100–110°C for the compression and metering sections (temperature controlled by air circulator). Collection of jerky-type extrudate was begun after 30 sec of a run to provide increased uniformity before it was cut manually with scissors into strips of desired lengths. The strips were chilled 30 min. (4°C) and weighed prior to being dried in a Blodgett® convectional oven (Type CTB-1, 1700 rpm) for 60 min. at 49°C. Following drying the extrudate

was reweighed and again chilled (4°C) 30 min. The extrudate was placed in resealable storage bags and held at 4°C until analyzed.

Physical and rheological properties

Shear strength. Ten samples from each run were sheared using a Warner-Bratzler® meat shear (Model D372-19, Instron Corp., Canton, MA) mounted on an Instron® Universal Testing Machine (Model 1120, Instron Corp., Canton, MA). The jerky-type samples were placed at right angles to the blade. Crosshead speed was 200 mm/min and full scale load was 5 kg. Energy (joules) required to shear, distance to peak force (mm) and height at peak force (mm) were determined.

Tensile strength. Ten samples from each run of the jerky-type product were utilized to measure the strength/energy necessary to pull the strips apart. Each end of the strip was firmly held by adjustable grips that were attached to the Instron® Universal Testing Machine (Model 1120, Instron Corp., Canton, MA) using attachments provided for the Warner-Bratzler meat shear. Energy (joules) required to pull strips apart, distance to peak force (mm) and height at peak force (mm) were determined.

Water activity (a_w). Chopped jerky-type extrudate was placed in plastic sample cups (5 cm × 14 mm) of a Rotronic® instrument (Model D2100, Rotronic Instrument Corp., Huntington, NY). Water activity was measured ±1.5% after an equilibration for 20–25 min using standard procedures for the instrument.

Colorimeter evaluation. A Minolta Chroma Meter (Model 310, Minolta Corp., Ramsey, NJ) was used to evaluate color of duplicate 2 cm × 4 cm jerky-type samples. Hunter L, a and b values were determined for each sample.

Sensory evaluation

Panelist selection. All faculty, staff and graduate students in the College of Agriculture and Home Economics at New Mexico State University were invited to participate in sensory panel evaluation of jerky-type food products. Personnel were asked to complete informational questionnaires and return them if they were interested in participating. The questionnaire included availability for training and evaluation, health status, snack food preferences and frequency of snack food consumption, including jerky. The 12 people who returned the questionnaire were invited to an organizational meeting. Four of the 12 declined to participate due to time constraints The remaining 8 then began sensory evaluation training.

Panelist training. Panelists met for 1 hr/wk for 10 wk prior to product evaluation. Training sessions were held in a temperature-controlled room under fluorescent lighting. Panelists were not separated during the first 5 wk of training to facilitate discussions. Portable, white, three-sided sensory booths were used to separate panelists during the second 5-wk of training. Each session was led by a moderator who directed activities but did not participate in evaluations or discussion.

Panelists evaluated aroma characteristics in two series of samples during Week 1. In the first series, panelists were asked to match each of six samples presented to one of the words on a list. In the second series, panelists were asked to describe a set of six samples using one or more of their own words. Products used in preparing the samples included spices such as black pepper and chile powder, rancid oil, burnt toast, potato starch and a combination of beef and chicken broth. After evaluation of each series of samples, panelists participated in a round-table discussion of aroma characteristics.

Panelists evaluated color in two series of samples during Week 2. In the first series, ability to identify red, blue, green and yellow was determined using samples prepared from distilled water and food coloring. A series of eight samples prepared from various combinations of food colorings in distilled water were then presented and panelists were asked to describe each color in their own words. This evaluation was followed by a round-table discussion of colors.

A list of sensory techniques for evaluating texture characteristics as well as a list of texture terms and definitions (Civille and Szczesniak, 1973; Stone, 1992) were distributed and discussed prior to texture evaluations conducted during Week 3. These lists were placed in each booth for use by panel members during all subsequent product evaluations. Panelists then evaluated hardness, adhesiveness and chewiness using standard rating scales (Szczesniak et al., 1962). Each evaluation was followed by a round-table discussion.

Flavor attributes were evaluated in two series of samples during Week 4 of training. In the first series, panelists were asked to match each of six samples to one word on a list. In the second series, panelists were asked to describe each of six samples using one or more of their own words. Products used in preparing the samples included rancid oil, burnt toast, potato starch, a combination of beef and chicken broth and spices such as black pepper, two types of chile powder and salt. Panelists participated in a round-table discussion after each evaluation.

Samples of extruded meat/potato jerky-type products, similar to those to be studied, were evaluated by panelists during Week 5. Two samples were presented to each panelist in 120 mL plastic sample cups with snap-on lids. Panel members first evaluated each sample for aroma, then appearance, then texture and finally flavor characteristics. After each evaluation on each sample, panelists discussed the attribute until agreement was reached on the major characteristics and terms for the characteristics. A scorecard consisting of line scales was then developed using the major characteristics and terms agreed upon by the panel. Line scales were 152 mm in length and anchored by two words placed 12.7 mm from each end representing the range of intensity for each attribute (Stone, 1992). All scales were developed so that the lower intensity term was the left anchor and the higher intensity term the right anchor.

During each 1 hr session in Weeks 6–10, panelists were served three to five samples of extruded meat/potato jerky-type products. Samples were presented one at a time in 120 mL plastic sample cups with snap-on lids. Panel members used the developed scorecard to evaluate each sample for aroma, appearance, texture and flavor characteristics. At the end of each session, panelists were asked to comment on problems encountered with the scorecard or evaluation method. As a result of these comments, the order of the characteristics presented on the scorecard was revised, samples were presented in plastic-lined bags rather than sample cups and further training on the proper way to use the scorecard was conducted.

Product evaluation. Products prepared from PDCB, MSC and C and seasoned with 0.5%, 1.0% and 1.5% AJI were evaluated. Samples were stored under refrigeration (4°C) until 72 hr before evaluation. At that time, each of the 2 cm × 4 cm samples was placed in a small, plastic-lined bag and left at room temperature (25°C) until evaluated. Six samples were served one at a time in randomized order to each panel member during each session in a temperature-controlled laboratory under fluorescent lighting using portable, white, three-sided sensory booths. Distilled water was used to cleanse palates between samples. Samples from the nine meat type/chile level treatments were randomized so that each jerky-type product was evaluated in duplicate by each panelist over the 3-wk evaluation.

Statistical analyses

Shear strength, tensile strength and water activity data were analyzed using analysis of variance. Duncan's Multiple Range test was used for separation of means.

Data from the colorimeter evaluation were analyzed using a multivariate split-plot analysis. Batches of jerky-type product made from the nine meat type by chile level combinations formed whole plots while the three 2 cm × 4 cm samples from each batch formed subplots. The Hunter L, a and b values measured on each sample were analyzed together using a multivariate analysis of variance. LSD values were used for mean separation.

The sensory panel data were analyzed using multivariate split-plot analysis within each sensory evaluation category (aroma, color, texture and flavor). Batches of jerky-type product formed whole plots while the 2 cm × 4 cm samples from each batch served to the panelists formed subplots. The multiple responses within sensory evaluation categories of aroma, color and flavor were analyzed together using a multivariate analysis of variance. Due to the large number of responses in the category of texture, a principal components factor analysis was performed to reduce the number of multiple responses. Four texture categories were derived. Factor 1 included smoothness, gumminess and grittiness; Factor 2 included hardness, cohesiveness during chewing and denseness; Factor 3 included adhesiveness and toothpacking; and Factor 4 included chewiness. The multivariate analysis of variance for a split-plot design was applied to the texture factor scores obtained from factor analysis. LSD values were used for mean separations within all four sensory evaluation categories.

RESULTS & DISCUSSION

Physical and rheological properties

Shear strength. A meat by chile interaction was detected in energy required to shear jerky-type products (Table 1). No differences ($p > 0.05$) in shear energy were detected between chile levels within the MSC or PDCB products. However, a substan-

Table 1—Shear and tensile forces for jerky-type products

Meat type/ Chile level	Shear forces: Energy (joules)	Shear forces: Ht at peak Force (mm)	Tensile Forces: Energy (joules)	Tensile Forces: Ht at peak Force (mm)
C				
0.5%	59.0[a]	26.5[a]	75.3[a]	34.1[a]
1.0%	69.3[a]	40.1[b]	72.8[a]	31.7[a]
1.5%	107.2[b]	54.8[c]	141.1[b]	59.1[b]
MSC				
0.5%	50.8[a]	24.9[a]	47.2[d]	28.4[a]
1.0%	56.6[a]	31.3[d]	64.9[a]	26.8[a]
1.5%	58.4[a]	32.0[d]	59.4[a]	25.0[a]
PDCB				
0.5%	98.2[b]	50.8[c]	125.1[c]	47.1[c]
1.0%	101.0[b]	54.9[c]	114.9[c]	59.0[b]
1.5%	86.1[b]	47.4[c]	72.2[a]	43.8[c]

[a-d] Numbers in the same column followed by the same letter are not significantly different ($p > 0.05$)

Table 2—Colorimeter evaluation of jerky-type products

Meat type/ Chile level	L values	a values	b values
C			
0.5%	39.06[a]	−24.37[a,b]	+10.53[a]
1.0%	39.65[a]	−24.98[a,c]	+10.75[a,b]
1.5%	40.13[a]	−24.92[a,c]	+11.30[b]
MSC			
0.5%	36.61[b]	−23.09[d]	+8.55[c]
1.0%	36.35[b]	−22.74[d]	+8.63[c]
1.5%	36.77[b]	−23.21[d,e]	+8.53[c]
PDCB			
0.5%	37.48[b]	−23.81[b,e]	+9.14[c,d]
1.0%	38.97[a]	−24.37[b,c]	+9.65[d,e]
1.5%	38.95[a]	−24.43[b,c]	+10.09[a,c]

[a-e] Numbers in the same column followed by the same letter are not significantly different ($p > 0.05$).

Table 3—Sensory evaluation of aroma and color characteristics of jerky-type products

Meat type/ Chile level	Aroma characteristics: Meaty (mm)	Aroma characteristics: Potatoey (mm)	Aroma characteristics: Chile (mm)	Color Characteristics: Walnut brown (mm)	Color Characteristics: Uniformity (mm)
C					
0.5%	9.2[a]	1.8[a]	2.4[a]	7.3[a]	9.3[a,b]
1.0%	8.3[a,b]	2.0[a,b]	2.4[a]	6.6[a,b,c]	8.4[a]
1.5%	8.3[a,b]	2.5[b,c]	2.3[a]	5.9[b]	8.5[a]
MSC					
0.5%	8.9[a,c]	2.2[a,b]	2.4[a]	10.7[c]	10.7[c]
1.0%	7.3[b]	2.2[a,b]	1.9[a]	10.0[c,d,f]	10.0[b,c]
1.5%	8.3[a,b]	2.3[a,b]	2.1[a]	10.5[c,d]	10.4[b,c]
PDCB					
0.5%	8.3[a,b]	1.9[a,c]	1.9[a]	10.4[c,d]	10.0[b,c]
1.0%	7.8[b,c]	2.6[b]	1.9[a]	9.5[d,e]	10.3[b,c]
1.5%	8.0[a,b]	1.7[a]	1.8[a]	9.0[e,f]	10.2[b,c]

[a-e] Numbers in the same column followed by the same letter are not significantly different ($p > 0.05$).

tial increase in shear energy was noted for products prepared from C when chile level increased from 1.0% to 1.5%.

No differences ($p > 0.05$) were detected in distance to peak force for any jerky-type products. However, a meat by chile interaction was detected for height at peak force (Table 1), which increased in products prepared from either MSC or C as AJI increased. No differences ($p > 0.05$) in height at peak force were detected between chile levels in product prepared from PDCB.

Tensile strength. A meat by chile interaction was detected in the tensile energy measurement (Table 1). Energy required to pull apart jerky-type product prepared from C changed little between the 0.5% and 1.0% AJI levels, but increased substantially when the AJI level increased to 1.5%. Tensile energy was not different ($p > 0.05$) for product prepared from PDCB at the 0.5% and 1.0% AJI levels, but decreased ($p < 0.05$) at the 1.5% AJI level. Energy required to pull apart jerky-type product prepared from MSC increased as AJI level increased from 0.5% to 1.0%, but was not different ($p > 0.05$) between the 1.0% and 1.5% AJI levels.

Table 4—Factor scores for sensory evaluation of texture characteristics of jerky-type products

Meat type/ Chile level	Factor 1[e]	Factor 2[f]	Factor 3[g]	Factor 4[h]
C				
0.5%	0.087[a,b]	−0.372[a,b]	0.048[a]	−0.349[a]
1.0%	0.044[a,b]	−0.095[a,c]	−0.007[a]	−0.264[a]
1.5%	−0.030[a,b]	0.689[d]	0.118[a]	0.514[b,c]
MSC				
0.5%	−0.121[a,b]	−0.824[e]	−0.298[a]	−0.581[a]
1.0%	−0.137[a]	−0.542[b,e]	0.048[a]	−0.498[a]
1.5%	−0.143[a]	−0.537[b,e]	0.120[a]	−0.398[a]
PDCB				
0.5%	0.048[a,b]	0.245[e]	−0.218[a]	0.224[b]
1.0%	0.156[b]	0.786[d]	−0.084[a]	0.829[c]
1.5%	−0.086[a,b]	0.834[d]	0.139[a]	0.240[b]

[a-d] Numbers in the same column followed by the same letter are not significantly different ($p > 0.05$).

[e] Factor 1 = smoothness, gumminess and grittiness

[f] Factor 2 = hardness, cohesiveness during chewing and denseness

[g] Factor 3 = adhesiveness, toothpacking

[h] Factor 4 = chewiness

No differences ($p > 0.05$) were detected in distance to peak force for any jerky-type products. However, a meat by chile interaction was again detected for the height at peak force measurement (Table 1). For product prepared from C, height at peak force decreased slightly as AJI level increased from 0.5% to 1.0% and then increased greatly when AJI level was increased to 1.5%. No differences ($p > 0.05$) in height at peak force were detected between chile levels in product prepared from MSC. Height at peak force increased as AJI level increased from 0.5% to 1.0% in product prepared from PDCB, but then decreased as AJI level increased to 1.5%.

There may be several explanations for effects of chile powder on textural properties of jerky-type products we noted in our results. Capsaicin and other capsaicinoids in chile peppers have many hydrogens in their structures which may participate in hydrogen bonding. In some instances, the chile powder may act as a binder, forming hydrogen bonds with solubilized proteins. This could result in stronger, more ordered structures and thus tougher textures. This binding effect seemed to be particularly evident in jerky-type samples prepared from C. The chile powder may also increase the water-holding capacity of blends through hydrogen bonding to water molecules. Retention of additional water could offset toughening typically associated with dehydration. This might explain decreases in shear and tensile forces which occurred with jerky-type product prepared from PDCB.

Water activity (a_w). No differences ($p > 0.05$) were found in water activity for any of the jerky-type products. Product prepared from MSC had the lowest a_w at 0.42. Jerky-type product prepared from PDCB had an a_w of 0.51 while that prepared from C had an a_w of 0.56.

Colorimeter evaluation. Hunter L values (Table 2) indicated that jerky-type products from MSC were darker ($p < 0.05$) than those prepared from C at each AJI level. This may be due in part to the higher levels of bone marrow and heme associated with MSC products (Fields, 1976). Heating results in the oxidation of the heme pigment to form the brown pigment hemin (Hultin, 1985). The presence of additional heme in MSC would result in darker color associated with jerky-type products prepared from that meat source. The PDCB jerky-type product containing 0.5% AJI was darker ($p < 0.05$) than the PDCB products containing 1.0% and 1.5% AJI, but was not different ($p > 0.05$) from any of the MSC products. The PDCB jerky-type products containing 1.0% and 1.5% AJI were not different ($p > 0.05$) from any of the C jerky-type products.

Table 5—Sensory evaluation of flavor characteristics of jerky-type products

	Flavor characteristic							
Meat type/Chile level	Meaty	Potatoey	Salty	Black Pepper	Chile Pepper	Sweet	Smoky	Bitter
C								
0.5%	7.8^{a}	2.2^{a,b}	4.5^{a}	8.2^{a}	3.9^{a,b}	3.4^{a}	4.2^{a,b}	3.0^{a,b}
1.0%	6.9^{a,b}	2.0^{a}	4.5^{a}	8.3^{a}	4.0^{a,b}	3.1^{a}	4.3^{a,b}	2.7^{a,b}
1.5%	7.3^{a,b}	2.8^{a,b}	5.4^{a}	10.5^{b}	5.6^{a}	3.3^{a}	4.0^{a,b}	2.8^{a,b}
MSC								
0.5%	7.5^{a,b}	3.1^{b}	4.9^{a}	6.0^{c}	3.1^{b}	3.8^{a}	3.6^{a}	2.5^{a,b}
1.0%	7.2^{a,b}	2.5^{a,b}	5.5^{a}	6.6^{c,d}	3.8^{a,b}	3.5^{a}	4.5^{a,b}	2.4^{b}
1.5%	6.5^{b}	2.5^{a,b}	5.1^{a}	7.8^{a,d}	4.8^{a,b}	3.6^{a}	4.3^{a,b}	3.1^{a}
PDCB								
0.5%	7.6^{a,c}	2.6^{a,b}	5.3^{a}	8.0^{a}	4.3^{a,b}	3.3^{a}	5.1^{b}	2.7^{a,b}
1.0%	6.8^{a,b}	2.3^{a,b}	5.0^{a}	8.2^{a}	3.6^{b}	3.5^{a}	3.7^{a}	3.1^{a}
1.5%	6.7^{b,c}	2.1^{a}	5.1^{a}	9.7^{b}	4.4^{a,b}	3.1^{a}	4.8^{a,b}	3.1^{a}

$^{a-d}$ Numbers in the same column followed by the same letter are not significantly different ($p>0.05$)

Table 6—Sensory evaluation of aftertaste characteristics of jerky-type products

	Aftertaste characteristic							
Meat type/Chile level	Meaty	Potatoey	Salty	Black Pepper	Chile Pepper	Sweet	Smoky	Bitter
C								
0.5%	6.4^{a,b}	1.6^{a}	4.1^{a}	8.9^{a,b}	4.2^{a}	3.2^{a,b}	3.5^{a}	2.9^{a}
1.0%	6.9^{a}	1.7^{a,b}	4.3^{a}	7.5^{a,c}	3.8^{a}	3.5^{a}	3.8^{a}	3.2^{a}
1.5%	6.2^{a,b}	2.3^{a,b}	5.0^{b}	10.5^{b}	5.2^{a}	3.1^{b}	3.6^{a}	2.9^{a}
MSC								
0.5%	6.7^{a,b}	2.7^{b}	4.4^{a}	6.1^{c}	3.5^{a}	3.9^{c}	4.0^{a}	2.9^{a}
1.0%	6.0^{a,b,c}	2.7^{b}	4.0^{a}	7.1^{a,c}	4.2^{a}	3.2^{a,b}	3.7^{a}	3.4^{a}
1.5%	5.8^{b,c}	2.6^{b}	4.3^{a}	8.0^{a}	4.4^{a}	3.3^{a,b}	3.5^{a}	3.4^{a}
PDCB								
0.5%	6.2^{a,b}	2.1^{a,b}	4.4^{a}	8.3^{a}	4.1^{a}	3.5^{a}	4.1^{a}	3.0^{a}
1.0%	5.9^{a,b,c}	1.7^{a,b}	4.3^{a}	9.0^{a,b}	4.4^{a}	3.3^{a,b}	3.2^{a}	2.9^{a}
1.5%	5.1^{c}	1.5^{a}	4.3^{a}	10.0^{b}	4.2^{a}	3.3^{a,b}	4.0^{a}	3.5^{a}

$^{a-c}$ Numbers in the same column followed by the same letter are not significantly different ($p>0.05$)

Table 7—Desirability of extruded jerky-type products prepared from meat and potato flour

	Average	Range	
Jerky-Type product	desirability score	High	Low
C			
0.5% chile	7.99	11.9	3.2
1.0% chile	8.06	11.9	3.3
1.5% chile	7.48	13.9	1.5
MSC			
0.5% chile	7.33	10.9	3.1
1.0% chile	7.77	11.3	3.6
1.5% chile	7.39	12.0	4.0
PDCB			
0.5% chile	8.68	12.7	4.7
1.0% chile	6.71	12.2	2.4
1.5% chile	6.61	11.5	2.0

Hunter a values (Table 2) for all products were consistently negative, indicating greenness. Both the seasoning blend and the potato flour used in preparation of the jerky-type products may have contributed to the greenness. Chile level did not affect the a values of jerky-type products within any meat type. The MSC jerky-type product containing 1.0% AJI was the least green of any product. Jerky-type products prepared from C tended to be the most green while those prepared from MSC tended to be the least green. The conversion of additional heme into hemin may have obscured the green characteristics contributed by other ingredients in products prepared from MSC.

Hunter b values (Table 2) indicated that all jerky-type products prepared from C and PDCB products containing 1.0% and 1.5% AJI were always more yellow than those from MSC. The C and PDCB jerky-type products containing 1.5% AJI were more yellow ($p < 0.05$) than their 0.5% chile counterparts, but no differences ($p > 0.05$) in yellowness due to chile level were detected for products made from MSC. The increased levels of bone marrow and heme typically associated with MSC products may have obscured any yellow contribution from AJI in such products.

Sensory evaluation

Aroma. Few differences ($p > 0.05$) were detected in aroma characteristics for jerky-type products (Table 3). Overall, meaty aroma was more prevalent than either potato or chile aromas in all samples. Jerky-type product prepared from C had the most intense meaty and chile aromas on average, while that from MSC had the most intense potato aroma on average. Jerky-type product prepared from PDCB had the least intense meaty, potato and chile aromas overall. The lack of change in chile aroma with increasing chile level was likely due to the lack of volatility of pungency principles found in chile (Lindsay, 1985).

Color. A difference ($p < 0.05$) in walnut brown color (Table 3) was detected by panelists between meat types. Product prepared from MSC was the darkest brown overall. This darker color was again probably due to conversion of additional heme to hemin in MSC products. Jerky-type product prepared from C was considerably lighter brown than PDCB or MSC jerky-type products.

Interactions were detected between panelists and meat and between panelists and chile level for color uniformity. This may indicate a lack of thorough mixing during blend preparation. A lack of strict temperature control during processing and/or drying may also have affected the products. In addition, a review of panelist data indicated that individual panel members consistently used the same part of the line scale for evaluation. However, one panelist always evaluated the products on the low end of the scale while the rest of the panel members tended to use the higher end of the scale.

Texture. Sensory panel texture data (Table 4) showed a meat by panelist interaction for Factor 1 (smoothness/grittiness). After discussion with panel members, it was apparent that further training in the area of grittiness was needed. A meat by chile interaction was detected by panelists for Factor 2 (hardness/denseness) for jerky-type product made from C. Sensory panel data indicated that as AJI increased, hardness of C jerky-type product also increased. This finding corresponded to data from

shear and tensile evaluations. It supported the possibility that chile powder had a binding effect in product made with C. Jerky-type product prepared from MSC was rated as least hard at each AJI level while that prepared from PDCB was rated as hardest. No differences ($p > 0.05$) were detected by panelists for Factor 3 (adhesive characteristics) due to meat type or AJI level. A meat by chile interaction was detected for Factor 4 (chewiness). Jerky-type product from MSC was always rated by the panel as least chewy at each AJI level, while PDCB jerky-type product tended to be the most chewy at each AJI level. Binding of additional water by capsaicinoid compounds to offset toughening due to dehydration may explain the chewier characteristics noted in products from PDCB. The binding properties of chile powder were again apparant in the large increase in chewiness noted for jerky-type product prepared from C as AJI level increased from 1.0% to 1.5%.

Flavor. No differences ($p > 0.05$) were detected in salty or sweet flavors during consumption due to meat type or AJI level (Table 5). AJI level affected ($p < 0.05$) black pepper flavor during consumption. Panelists detected greater black pepper flavor as AJI level increased regardless of meat type. Although chile pepper flavor tended to increase slightly with increasing AJI, no differences ($p > 0.05$) were detected within a meat type. The increasing black pepper flavor combined with the lack of change in chile pepper flavor with increasing AJI level may have been due to panelists evaluating pungency rather than flavor. Also pungency carry-over between samples probably influenced panelist evaluations.

No differences ($p > 0.05$) were detected in chile pepper, smoky or bitter aftertastes (Table 6). Black pepper aftertaste tended to increase with increasing AJI level, but chile pepper aftertaste remained fairly steady with increasing AJI level. This discrepancy may also have indicated that panelists evaluated pungency rather than overall flavor as well as pungency carry-over.

Overall desireability. Average desireability scores and corresponding ranges (Table 7) showed product from PDCB containing 0.5% chile were rated as the most desireable, followed by those from C containing 1.0% chile. Wide ranges in desireability scores were noted for all jerky-type products. Average desireability scores, however, indicated that acceptable jerky-type products could be prepared by extrusion of seasoned meat/potato blends.

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Detectable Odor Thresholds of Selected Lipid Oxidation Compounds in a Meat Model System

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ABSTRACT

Organic compounds, pentanal, hexanal, t-2-hexenal, t-2-heptanal, t-2-octenal, and t,t-2,4-decadienal, were added individually to lean ground beef. Detectable odor threshold (DOT) was determined by a 10-member sensory panel using triangle tests. Descriptive analysis using category scales measuring intensity was used to assess odor intensities contributed by specific compounds in an effort to relate quantitative differences in qualitative characteristics to oxidized beef. DOT for pentanal was 2.67 ppm; hexanal was 5.87 ppm; heptanal was 0.23 ppm; t-2-hexenal was 7.87 ppm; t-2-octenal was 4.20 ppm; and t,t-2,4-decadienal was 0.47 ppm. Common terms used to describe odor of meat containing added compounds were rancid, painty, and herbal.

Key Words: odor threshold, lipid oxidation, meat, model system

INTRODUCTION

PERCEIVED COOKED BEEF AROMA originates from a variety of volatile compounds formed during cooking. Lipid oxidation products result in off-odors and off-flavors in cooked beef (St. Angelo, 1992). Determining detection odor threshold levels of such compounds in their original matrices is necessary in order to establish beef quality standards and enhance consumer acceptability (Shahidi et al., 1986). If the odor detection threshold concentration of a particular compound is lower than its usual concentration in that product, the compound would contribute to the aroma of the product (Guadagni et al., 1968).

Differences in odor detection thresholds of several lipid oxidation products (aldehydes) have been reported using different media (air, water, milk, vegetable oil, mineral oil, paraffin oil, and gelatin); odor thresholds differed widely among different media (Fazzalari, 1978; Lillard et al., 1962; Lillard and Powers, 1975; Salo, 1970; Vega and Brewer, 1994). These studies indicated the different degrees of variation the matrix may cause for odor thresholds.

Using gas chromatography-mass spectrometry, over 600 volatile compounds have been identified in beef, including hydrocarbons, aldehydes, ketones, alcohols, carboxylic acids, esters, lactones, furans, pyridines, and pyrazines (Shahidi et al., 1986; Wu and Brewer, 1994). Aldehydes are significant contributors to the odor of cooked beef. Alkanals, alkenals, and alkadienals are responsible for a wide range of oxidized odors in beef (Love and Pearson, 1971; Wu and Brewer, 1994).

Most studies on the sensory characteristics of oxidized meat systems have measured the degree of oxidation and identified and quantified the oxidation products in the sample (usually in headspace gases). These were then correlated with sensory data derived from descriptive panel evaluation of the same samples. However, we do not know at what levels such substances can be detected or what the impact of these individual substances may be on sensory characteristics of the product.

Studies have reported descriptors for oxidized beef systems, including astringent, bitter, grassy, metallic, musty, sweet, putrid, sour, and stale using hedonic rating scales. Descriptors have been divided into two groups, "desirable" and "undesirable" notes (Berry et al., 1980). Using Quantitative Descriptive Analysis, Melton et al. (1982) reported ground beef descriptors, including fishy, metallic, milky, oily, sheepy, sweet, and stale. MacLeod and Coppock (1978) reported cooked beef aroma descriptors, including animal, blood, fragrant, sharp, pungent, and sickly. Many off-odor compounds form during autoxidation of unsaturated fatty acids, including oleic, linoleic, linolenic, and arachidonic acids. Depending on the location of the susceptible carbon, position, number of double bonds, and location of oxygen addition as well as the point of hydrocarbon chain β-scission, particular end products could be expected to be generated from autoxidation. Pentanal, hexanal, heptanal, t-2-hexenal, t-2-octenal, and t,t-2,4-decadienal may be formed from the decomposition of alkyl hydroperoxides of linolenic acid (Selke et al., 1978). MacNeil and Dimick (1970) reported that trained panelists rejected samples of stored poultry skin in which high levels of aldehydes, including 2-enals and 2,4-dienals, were present. Gas chromatographic and sensory analysis of fresh cooked beef, stored at 4°C and reheated after 24 hr, showed that pentanal, hexanal, heptanal, t-2-hexenal, t-2-octenal, and t,t-2,4-decadienal were present in parts per million and contributed notably to off-odors (St. Angelo et al., 1987). In addition to the increase in lipid oxidation products, which generate off-odors, often described as painty, fishy, and herbal, a loss of desirable odors, such as meaty, fatty, and oily, occurs (St. Angelo, 1992). Lipid oxidation products (in parts per million) greatly obscure the pleasant aroma contributors usually present (in parts per billion) (Liu et al., 1992; St. Angelo et al., 1987; Vercellotti et al., 1987). No attempts have been made to determine the sensory characteristics of cooked and raw beef after lipid oxidation compounds were added at known concentrations. This is needed to establish marker substances for lipid oxidation known to contribute to odor/flavor deterioration, and levels at which such deteriorative changes may be expected.

Our objectives were to determine detectable odor thresholds of selected lipid oxidation compounds in cooked ground beef, to determine odor descriptors for cooked and raw ground beef with such compounds added and to describe the odors of ground beef containing suprathreshold levels of selected ones.

MATERIALS & METHODS

ORGANIC COMPOUNDS, including GC grade pentanal, hexanal, t-2-hexenal, t-2-heptanal, t-2-octenal, and t,t-2,4-decadienal, were obtained from

Table 1—Detection odor threshold group geometric means for selected aldehydes in lean ground beef

	Geometric means[a]		Lowest threshold value (ppm)	Highest threshold value (ppm)
	Threshold (M)	Threshold (ppm)		
Pentanal	3.0×10^{-8}	2.67	1.41	5.55
Hexanal	5.8×10^{-8}	5.87	2.37	36.81
Heptanal	0.2×10^{-9}	0.23	0.004	0.484
t-2-Hexenal	8.0×10^{-8}	7.87	2.04	10.62
t-2-Octenal	3.3×10^{-8}	4.20	1.12	26.30
t,t-2,4-Decadienal	0.3×10^{-9}	0.47	0.013	3.08

[a] n/geometric mean = 180.

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Table 2—Effect of cooking treatment on sensory characteristics of ground beef containing added hexanal[z,y]

Descriptor	Raw, above[x]	Cook, below[w]	Cook, above[x]	SEM[v]
Putrid	1.20[a]	0[c]	0.77[b]	0.11
Sour	1.33[b]	2.17[a]	1.87[a]	0.09
Sweaty	1.63[a]	0[b]	2.00[a]	0.10
Rancid	3.03[a]	0[b]	3.07[a]	0.16
Animal	1.10[b]	3.10[a]	1.50[b]	0.16
Blood	1.90[a]	0[b]	0[b]	0
Fatty	1.40[b]	3.00[a]	1.60[b]	0.14
Oily	0.67[c]	3.13[a]	1.80[b]	0.14
Meaty	1.00[c]	3.13[a]	1.67[b]	0.14
Raw meat	3.40[a]	0[b]	0[b]	0.18
Fishy	1.70[a]	0[c]	1.27[b]	0.10
Painty	4.20[a]	0[c]	3.43[b]	0.22
Herbal	3.43[a]	0[b]	3.43[a]	0.18

[z] Category scale: 0 = absent, 5 = extremely high.
[y] n/LS mean = 30.
[x] Concentration of compound added = 55 ppm.
[w] Concentration of compound added = 55 ppb.
[v] SEM = standard error of the mean.
[a,b,c] Means in a row with different superscript letters are different ($p<0.05$).

Aldrich Chemical Company (Milwaukee, WI). Stock solutions were prepared in distilled water (22°C) in parts per million concentrations, flushed with nitrogen, capped, and stored at 4°C in amber glass bottles.

Fresh lean beef top round (35 kg) was obtained from the University of Illinois Meat Science Laboratory, double-wrapped in freezer paper, and frozen at −18°C in 454-g aliquots. Lean beef was thawed (4°C for 4 hr), ground (0.50-cm plate), and mixed (1:1) with distilled water (blank), or with organic stock solutions, to predetermined concentrations. Samples (15 g) were placed in 25-mL amber vials and sealed. Samples were cooked in a circulating water bath to 70°C (internal temperature) and maintained at 45°C in a water bath for odor analysis. Samples were cooked 15-20 min before odor evaluation.

Sensory analysis

Threshold tests. A 10-member experienced panel, aged 18 to 35 years (x = 25), all women, non-smokers, was trained (10, 1-hr sessions) using preliminary triangle testing to determine concentration ranges needed for stock solutions. In order to familiarize the panel with the characteristic odor of each compound, panelists were asked to describe the aroma of the added compounds in meat at different concentrations ranging from 10 ppb to 100 ppm. Panelists were acquainted with the typical odors of oxidized foods (rancid oils, pork and beef). The panel then generated a list of odor descriptors for oxidized beef by group consensus selecting descriptors from a composite list of all descriptors used by individual panelists for describing the odor of oxidized meat samples. Judges used pentanal (50 ppm), hexanal (220 ppm), t-2-hexenal (120 ppm), heptanal (100 ppm), t-2-octenal (150 ppm) and t,t-2,4-decadienal (110 ppm) to practice using the descriptors. These concentrations were selected based on concentrations reported in meat systems.

Test samples for threshold evaluation were presented to judges in increasing order of concentration. Judges were given 6 sets of 3 samples, asked to smell the samples, and select the odd sample based only on odor differences. Judges were instructed to pause 10-20 sec between samples. Panelists performed 10 replicates of samples containing each compound at each concentration over 6 mos. Olfactory evaluation was performed in the sensory laboratory, at 22°C, and 60% relative humidity; fans were used to create positive air pressure.

Odor description

Panelists were asked to rate the aroma using the list of odor descriptors established during the training session. A 5-point category scale (0 = absent, 1 = slight, 2 = mild, 3 = moderate, 4 = high, and 5 = extremely high) was used to rate cooked samples containing each of the compounds (individually) at concentrations below odor threshold (55 ppb) and above odor threshold (55 ppm), and for uncooked samples (55 ppm). Panelists were provided with samples of cooked and raw beef with no added compounds, along with standards used for training as reference points. Three replications of odor intensity evaluation were performed.

Statistical analysis

PROC NLIN (SAS Institute, Inc., 1993) was used to analyze triangle test threshold data for each aldehyde and panelist individually by fitting nonlinear regression models using the least squares method (ASTM, 1992). In order to calculate detectable odor thresholds for each panelist, the percent of correct responses (above chance) was calculated at each concentration for each compound. The nonlinear regression model (SAS Institute, Inc., 1993) fits the best curve to the data points. The concentration at which panelists gave the correct response 50% of the time (above chance) was considered to be the detectable odor threshold for a specific panelist for that specific compound. Geometric means were calculated for group detectable odor thresholds.

LS means were calculated for the intensity of each descriptor for each compound by treatment (raw above threshold, cooked below threshold, and cooked above threshold) using the General Linear Model (SAS, 1993). Means which were significantly different ($p < 0.05$) were separated using probability of difference (SAS Institute, Inc., 1993).

RESULTS & DISCUSSION

DETECTION ODOR THRESHOLD DIFFERENCES were three orders of magnitude for pentanal, hexanal, t-2-hexenal, t-2-octenal, and two orders of magnitude for heptanal and t,t-2,4-decadienal in a beef system compared to the same compounds in a gelatin system (Vega and Brewer, 1994) (Table 1). Cooked beef DOT values ranged from 0.23 ppm for heptanal to 7.87 ppm for t-2-hexenal. DOT values of some aldehydes reported in beef include methional (6.1 ppm), phenylacetaldehyde (0.94 ppm), and nonanal (7.6 ppm) (Wick et al., 1967). DOT values we determined were in the ppm range and comparable to those reported in other studies. The orders of magnitude of difference among DOT values in liquid systems (water and oil), gels, and a beef system may be due to interactions between proteins and volatile aldehydes in the beef matrix during cooking. They may also relate to the variety and large number of volatiles in raw and cooked beef when compared to the relatively few in water, oil, and gel systems.

Hexanal and t-2-hexenal, which exhibited higher DOT values in our study, have been shown to bind more readily to protein than do the other aldehydes evaluated. Binding increases with protein denaturation (Solms et al., 1973). Aldehydes may react with side chains of lysine, arginine, methionine, and tyrosine to form Schiff bases (Kinsella, 1982; Ory and St. Angelo, 1982). Hydrophobic interactions and hydrogen bonds between proteins and similar aldehydes have been reported; as the protein unfolds, more hydrophobic groups are exposed to the media, increasing the potential for aldehyde binding (Arai et al., 1970). When aldehydes are bound to protein, they behave as non-volatile compounds which reduces their concentrations in the headspace (Arai, 1980). Experiments with canned beef containing added arginine and lysine had reduced aldehyde concentrations in the headspace. During heating, protein unfolding increased, enhancing the binding of aldehydes with protein (Sydow, 1975). In addition, meat is a much more viscous system, especially at higher temperatures (70°C) than gelatin, water, or oil. The higher viscosity may restrict molecular movement through the matrix to the headspace.

The most frequent odor descriptors for cooked beef samples containing each of the compounds added at concentrations below detection threshold were fatty, oily, and meaty, typical of fresh cooked beef (Tables 2–6, Fig. 1). In cooked beef samples containing suprathreshold concentrations of those compounds, the pleasant cooked beef odor characteristics (fatty, oily, and meaty) were overpowered by the negative odor descriptors, such as rancid, painty, and herbal (Tables 2–6, Fig. 1). These odor descriptors are associated more with oxidized systems than with fresh cooked beef systems (St. Angelo et al., 1990). The most common descriptors for raw beef with suprathreshold concentrations of each of the compounds were rancid, raw meat, painty, and herbal (Tables 2–6). When beef has not been cooked, aldehydes are less likely to be bound to protein. The original odor characteristics of the compounds are more easily recognized by sensory panelists, probably because their headspace concentration is much higher (Arai, 1980; Arai et al., 1970; Solms et al., 1973). During cooking, many compounds combine with sulfur

Table 3—Effect of cooking treatment on sensory characteristics of ground beef containing added heptanal[z,y]

Descriptor	Raw, above[x]	Cook, below[w]	Cook, above[x]	SEM[v]
Putrid	1.34[a]	0[c]	0.64[b]	0.10
Sour	1.20[c]	2.83[a]	1.73[b]	0.11
Sweaty	1.93[a]	0[b]	2.00[a]	0.10
Rancid	3.47[a]	0[b]	3.27[a]	0.8
Animal	0.37[b]	3.77[a]	0.64[b]	0.19
Blood	0.61[a]	0[b]	0[b]	0.07
Fatty	0.90[c]	3.90[a]	1.10[b]	0.17
Oily	0.90[c]	3.80[a]	1.40[b]	0.15
Meaty	0.90[c]	3.80[a]	1.40[b]	0.16
Raw meat	4.37[a]	0[b]	0[b]	0.21
Fishy	1.80[a]	0[b]	1.53[a]	0.12
Painty	4.23[a]	0[c]	3.73[b]	0.22
Herbal	4.00[a]	0[b]	4.00[a]	0.20

[z] Category scale: 0 = absent, 5 = extremely high.
[y] n/LS mean = 30.
[x] Concentration of compound added = 55 ppm.
[w] Concentration of compound added = 55 ppb.
[v] SEM = standard error of the mean.
[a,b,c] Means in a row with different superscript letters are different ($p<0.05$).

Table 4—Effect of cooking treatment on sensory characteristics of ground beef containing added t-2-hexenal[z,y]

Descriptor	Raw, above[x]	Cook, below[w]	Cook, above[x]	SEM[v]
Putrid	1.44[a]	0[c]	0.84[b]	0.12
Sour	1.20[b]	2.30[a]	1.90[a]	0.08
Sweaty	1.40[b]	0[c]	2.30[a]	0.12
Rancid	3.77[a]	0[b]	3.53[a]	0.19
Animal	1.14[b]	2.53[a]	1.27[b]	0.13
Blood	0.11[a]	0[a]	0[a]	0.03
Fatty	0.71[c]	2.60[a]	1.50[b]	0.14
Oily	1.03[c]	3.07[a]	1.60[b]	0.14
Meaty	0.83[c]	3.07[a]	1.33[b]	0.14
Raw meat	3.87[a]	0[b]	0[b]	0.20
Fishy	1.97[a]	0[c]	1.23[b]	0.11
Painty	2.87[a]	0[c]	2.43[b]	0.20
Herbal	4.00[a]	0[b]	4.00[a]	0.20

[z] Category scale: 0 = absent, 5 = extremely high.
[y] n/LS mean = 30.
[x] Concentration of compound added = 55 ppm.
[w] Concentration of compound added = 55 ppb.
[v] SEM = standard error of the mean.
[a,b,c] Means in a row with different superscript letters are different ($p<0.05$).

Table 5—Effect of cooking treatment on sensory characteristics of ground beef containing added t-2-octenal[z,y]

Descriptor	Raw, above[x]	Cook, below[w]	Cook, above[x]	SEM[v]
Putrid	1.20[a]	0[c]	0.71[b]	0.11
Sour	1.13[c]	3.00[a]	1.60[b]	0.11
Sweaty	1.63[b]	0[c]	2.00[a]	0.10
Rancid	3.87[a]	0[b]	3.87[a]	0.21
Animal	0.54[c]	3.73[a]	1.07[b]	0.20
Blood	0.14[a]	0[b]	0[b]	0.04
Fatty	0.21[c]	4.20[a]	0.61[b]	0.21
Oily	1.16[b]	4.26[a]	1.13[b]	0.17
Meaty	1.13[b]	4.27[a]	1.00[b]	0.17
Raw meat	4.00[a]	0[b]	0[b]	0.20
Fishy	1.40[a]	0[c]	1.27[b]	0.10
Painty	3.93[a]	0[c]	4.00[a]	0.21
Herbal	4.00[a]	0[b]	4.00[a]	0.20

[z] Category scale: 0 = absent, 5 = extremely high.
[y] n/LS mean = 30.
[x] Concentration of compound added = 55 ppm.
[w] Concentration of compound added = 55 ppb.
[v] SEM = standard error of the mean.
[a,b,c] Means in a row with different superscript letters are different ($p<0.05$).

Table 6—Effect of cooking treatment on sensory characteristics of ground beef containing added t,t-2,4-decadienal[z,y]

Descriptor	Raw, above[x]	Cook, below[w]	Cook, above[x]	SEM[v]
Putrid	1.04[a]	0[c]	0.54[b]	0.10
Sour	1.93[b]	3.00[a]	2.20[b]	0.10
Sweaty	1.30[b]	0[c]	2.23[a]	0.12
Rancid	2.87[a]	0[b]	2.80[a]	0.19
Animal	0.67[c]	3.67[a]	1.34[b]	0.18
Blood	1.11[a]	0[b]	0[b]	0.09
Fatty	1.70[b]	3.80[a]	1.70[b]	0.17
Oily	0.90[c]	3.90[a]	1.80[b]	0.16
Meaty	0.60[c]	3.90[a]	1.80[b]	0.17
Raw meat	4.43[a]	0[b]	0[b]	0.22
Fishy	2.10[a]	0[c]	2.27[a]	0.15
Painty	3.00[a]	0[c]	4.00[b]	0.22
Herbal	4.60[a]	0[c]	2.20[b]	0.20

[z] Category scale: 0 = absent, 5 = extremely high.
[y] n/LS mean = 30.
[x] Concentration of compound added = 55 ppm.
[w] Concentration of compound added = 55 ppb.
[v] SEM = standard error of the mean.
[a,b,c] Means in a row with different superscript letters are different ($p<0.05$).

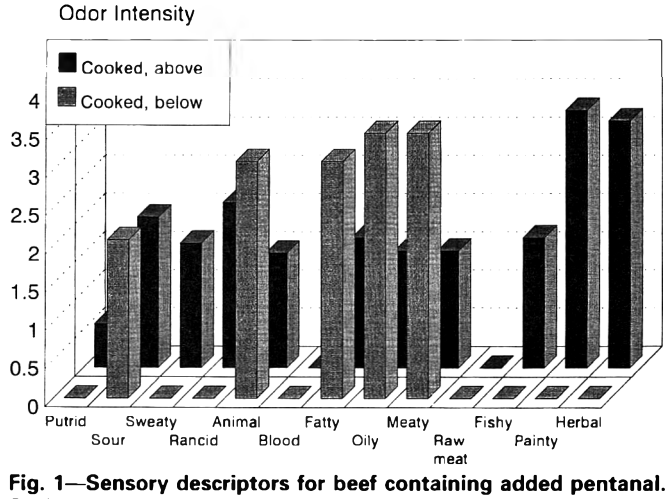

Fig. 1—Sensory descriptors for beef containing added pentanal. Scale: 0 = none, 5 = intense.

and nitrogen compounds to produce characteristic cooked meat odors (St. Angelo et al., 1987). Cooked beef contains more volatiles than raw beef (St. Angelo, 1992), increasing the additive effect of sub-threshold concentrations of any other volatile compounds associated with cooked beef aroma (Guadagni et al., 1963). The more volatiles present in the headspace, the more difficult it became to distinguish and detect any one individually.

The predominant descriptors (more than 3.0, scale = 1–5) used by sensory panelists for cooked meat containing above threshold concentration of pentanal were painty and herbal (Fig. 1). For hexanal, descriptors were painty, herbal, and rancid (Table 2); for heptanal, descriptors were herbal, painty, and rancid (Table 3); for t-2-hexenal, descriptors were herbal and rancid (Table 4); for t-2-octenal, descriptors were painty, herbal, and rancid (Table 5); and for t,t-2,4-decadienal, the predominant descriptor was painty (Table 6). The consistent use of the descriptors painty, herbal, and rancid confirmed that this group of aldehydes constitutes part of the group that causes the oxidized odor of beef.

When ground beef containing above or below threshold concentrations of pentanal was cooked, significant differences in intensity occurred for most sensory descriptors (Fig. 1). Cooked samples containing below threshold concentrations of pentanal had higher ($p < 0.05$) animal, fatty, oily, and meaty odors than samples containing above threshold concentrations. Putrid, sweaty, rancid, fishy, painty, and herbal odor intensities were higher in samples containing above threshold concentrations; fatty, oily, and meaty odor intensities were lower in above threshold concentration samples. This trend was consistent for hexanal, t-2-hexenal, heptanal, t-2-octenal, and t,t-2,4-decadienal.

A consistent trend for all compounds was the reduction of characteristic cooked meat odors (fatty, oily, and meaty) in the presence of above threshold added compounds. It appeared that the less acceptable odors (rancid, sweaty, fishy, painty, putrid, and herbal) obscured the characteristic meat odors.

Other studies to derive cooked beef odors reported odor descriptors similar to those in our study. Panelists found significant

rancidity differences ($p < 0.05$) between freshly cooked beef, stored beef (1 day, 4°C), and reheated beef (St. Angelo et al., 1987). Character notes used by trained sensory panels to describe oxidized beef systems have included cardboardy, rancid, stale, and metallic (Civille and Dus, 1992; Johnsen and Civille, 1986).

CONCLUSIONS

THE LARGER NUMBER OF VOLATILES in meat increased odor detection thresholds of these compounds from parts per billion (ppb) concentration range in liquid media, such as water and oils, to parts per million (ppm) concentrations in beef. Cooked beef DOT values ranged from 0.23 ppm for heptanal to 7.87 ppm for t-2-hexenal. DOT cooked beef values increased: heptanal > t,t-2,4-decadienal > pentanal > t-2-octenal > hexanal > t-2-hexenal. This study provides an odor quality scale with a list of descriptors and standards that could be used in the evaluation of beef products undergoing lipid oxidation. Selected lipid oxidation compounds at concentrations above detectable odor threshold confirmed them to be responsible for some of the oxidized odors of ground beef.

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Gamma Irradiation Effects on Thiamin and Riboflavin in Beef, Lamb, Pork, and Turkey

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ABSTRACT

A study was made of the loss of thiamin and riboflavin due to gamma irradiation of beef, lamb and pork *longissimus dorsi*, turkey breast and leg muscles. Thiamin losses averaged 11%/kiloGray (kGy) and riboflavin losses 2.5%/kGy above three kGy. The rate of loss of thiamin in beef was higher than that in lamb, pork and turkey leg, but not turkey breast, with losses of 16%/kGy in beef and 8%/kGy in lamb. The rate of thiamin loss was not related to sulfhydryl, protein, moisture, fat or water content, pH or reducing capacity by redox titration. Loss of riboflavin was not different among species. Any detriment from such slight losses would seem to be more than compensated by the advantage of controlling bacteriological contamination by irradiation processing.

Key Words: meats, gamma irradiation, poultry, thiamin, riboflavin

INTRODUCTION

THE USE OF GAMMA IRRADIATION at doses of 0.3 to 1.0 kGy has been approved for elimination of trichina in pork (CFR, 1993a) and of 1.5 to 3.0 kGy for pasteurization of chicken (CFR, 1993b). With regard to outbreaks of *E. coli* 0157:H7 in ground beef, food irradiation could be a partial solution for ensuring the safety of hamburgers (FCN, 1993). Use of low-dose irradiation has been proposed for general approval for all meats (Merritt and Taub, 1983). The basis for the proposal is the concept of "chemiclearance" which states that the effects of irradiation are primarily based on the received dose. It also indicates it is possible to extrapolate results from higher to lower doses, and that the irradiation of similar substrates would have comparable results. We have previously reported the effects of gamma radiation on the loss of vitamins B_1, B_2, B_6, B_{12} and niacin in pork chops and chicken breasts (Fox et al., 1989) and the loss of thiamin in ground beef, chicken and pork (Fox et al., 1993). Our current objective was to test the hypothesis that the effect of low-dose gamma radiation on thiamin and riboflavin would be the same in muscle tissues regardless of species. We tested the effects of gamma radiation on beef, lamb, pork and turkey under identical conditions. For turkey, both breast and leg muscles were studied. The breast consists primarily of white muscle fibers and leg predominantly of red fibers thus the two have differing physiological properties (Cassens and Cooper, 1971). In addition to thiamin and riboflavin, we studied other muscle tissue components, specifically free sulfhydryl groups, pH and total reducing capacity.

MATERIALS & METHODS

Meats

All meats were purchased locally on the day after slaughter. Pork was purchased from Leidy of Soudertown, PA, beef (steer) from Carl Venezia of Conshohocken, PA, lamb and turkey from C. Fehl's of Spring House, PA. The meats were the *longissimus dorsi* of the mammals, and the breast and all of the leg muscles of the turkey. An attempt was made to keep the number of individual animals to a minimum but it was not always possible. The beef came from at least three animals, the pork from three, the lamb from six or more and the turkey from ten. All muscles were carefully trimmed of fat, then diced into cubes and frozen on dry ice. The meat was then pulverized in a Hobart silent cutter to yield a homogeneous powder which formed a smooth paste when thawed, suitable for precisely determining changes in muscle components. This preparation has been compared with ground and whole muscle samples and, as far as vitamin loss is concerned, yielded the same results, only with greater precision. The powder was allowed to thaw, packaged into Cryovac E-300 poultry bags (oxygen transmission 4000 $cm^3/m^2/24$ hr, 1 atm at 22.8°C) and hung from racks in the center of the radiation field for irradiation treatment. Most determinations were performed immediately, but samples for proximate analyses required storage. These samples were packaged *in vacuo* into high-barrier polyiminocaproyl/aluminum/polyethylene foil packages (Thayer et al., 1987) and held at −50°C until analyses were performed.

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Gamma-ray source

The gamma-ray source was a ^{137}Cs unit built by Lockheed Corporation, Marietta, Georgia, operating at a level of 0.108 kGy/min. Reference dosimeters from the National Physical Laboratory, Middlesex, United Kingdom, were used to calibrate the source. Dosimetry and dose distribution for the source have been described by Shieh et al. (1985). Samples were hung in bags from a stainless steel rack in the center of the gamma-ray field and exposed to doses of 0.234, 0.468, 0.937, 1.875, 2.812, 5.624, and 9.374 kGy. The temperature of the radiation chamber was maintained at 5 ± 0.5°C during irradiation by injecting the gas phase from liquid nitrogen. Sample temperature was monitored continuously during irradiation.

Thiamin

Thiamin was determined as previously described including blending of sample with 2% trichloroacetic acid (TCA), heating, centrifugation and determination by flow injection (FID) (Fox et al., 1992). Thiamin was oxidized to thiochrome by $K_3Fe(CN)_6$ and the concentration of the thiachrome was measured by its fluorescence. $\lambda_{excitation}$ = 365 nm, $\lambda_{emission}$ = 460 nm.

Riboflavin

Riboflavin was determined in the TCA extracts by FID from its fluorescence, $\lambda_{excitation}$ = 450 nm, $\lambda_{emission}$ = 530 nm.

Reducing capacity, sulfhydryl and pH

Total reducing capacities were determined by titration with dichlorophenolindophenol (DCPIP) (Fox et al., 1993). The sulfhydryl content was determined by use of Ellman's Reagent according to the method of Beverage et al., 1974. The pH was determined by placing a Ross (Orion Research, Inc., Boston, MA) combination pH electrode in contact with the meat.

Proximate analysis

The water and fat were determined by CEM methods (AOAC, 1990) with one modification. After determination of moisture content, the samples for fat determination were blended with 100 mL methylene chloride and slurries were quantitatively transferred to filter paper in a Buchner funnel and the methylene chloride removed by suction. Protein and ash were determined using CEM microwave furnaces: MAS-300 furnace for ash (CEM, 1989; Zhang and Dotson, 1994) and Kjel-FAST furnace for Kjeldahl digestion (CEM, 1987). After sulfuric acid/H_2O_2 digestion in the CEM oven, the alkalization and distillation of ammonia were carried

Table 1—Analysis of covariance of losses of thiamin in beef, lamb, pork and turkey with gamma irradiation as a covariate. General Linear Models Procedure, SAS. (Model: Ln [% thiamin] = Ln [% thiamin]$_0$ + slope × dose)

Source	DF	Sum of squares	Mean square	F value	Pr > F
Model	9	15.6	1.73	19.9	0.0001
Error	107	9.3	0.087		
Corrected total	116	24.9			

R-square	C.V.	Root MSE	LNT mean
0.626	6.90	0.295	4.29

Source	DF	Type I SS	Mean square	F value	Pr > F
D	1	13.9	13.90	159.76	0.0001
Species	4	0.756	0.189	2.17	0.077
D* Species	4	0.915	0.229	2.63	0.038

Parameter	Estimate	T for H0: parameter=0	Pr > ITI	Std. error of estimate
INTERCEPT	4.554 B	57.0	0.0001	0.80
Beef slope	−0.176	−7.7	0.0001	0.0229
Lamb slope	−0.083	−4.20	0.0001	0.0196
Pork slope	−0.104	−5.30	0.0001	0.0196
Tbre* slope	−0.119	−6.07	0.0001	0.0196
Tleg* slope	−0.103	−5.28	0.0001	0.0196

Contrast	DF	Contrast SS	Mean square	F value	Pr > F
Beef vs Pork	1	0.837	0.837	9.62	0.0025
Beef vs Lamb	1	0.493	0.493	5.67	0.019
Beef vs Tbre*	1	0.307	0.307	3.53	0.063
Beef vs Tleg*	1	0.500	0.500	5.75	0.018

* Tbre, turkey breast; Tleg, turkey leg.

Table 2—Analysis of covariance of the losses of riboflavin in beef, lamb, pork and turkey with gamma irradiation as a covariate. General Linear Models Procedure, SAS. (Model: [riboflavin] = [riboflavin]$_0$ + slope × dose

Source	DF	Sum of squares	Mean square	F value	Pr > F
Model	14	1395.56	99.76	0.90	0.56
Error	104	11482.44	110.4		
Corrected total	118	12878.01			

R-square	C.V.	Root MSE	T mean
0.108368	10.71951	10.50753	98.0224971

Source	DF	Type I SS	Mean square	F value	Pr > F
D	1	625.02	625.0	5.66	0.019
Species	4	157.42	39.4	0.36	0.84
D* Species	4	233.61	58.4	0.53	0.72
D*D	1	107.14	107.1	0.97	0.33
D*D* Species	4	272.38	68.1	0.62	0.65

Parameter	Estimate	T for H0: parameter=0	Pr > ITI	Std. error of estimate
INTERCEPT	103.07 B	29.24	0.0001	3.53
Beef slope	−3.24	−1.28	0.204	2.54
Lamb slope	−3.95	−1.53	0.130	2.59
Pork slope	1.95	0.75	0.452	2.59
Tbre* slope	−2.17	−0.84	0.446	2.59
Tleg* slope	−1.98	−0.77	0.4455	2.59

* Tbre, turkey breast; Tleg, turkey leg.

out in a Kjeltec apparatus (AOAC, 1990). The ammonia was trapped in boric acid and titrated with 0.1N HCl to a bromcresol green/methyl red endpoint.

Mathematical analyses

Raw data were converted into regressions or subjected to analysis of covariance (ANCOVA) using appropriate procedures in the SAS software system (SAS Institute, Inc., 1987). Regressions were determined by the NLIN procedure and the ANCOVA by the GLM program. Regression coefficients were calculated from the following equation which expresses the dependence of the observed rate constant on the reductant concentration:

$$k_{observed} = k_o / (1 + k_r \times [R]) \quad (1)$$

RESULTS

Thiamin loss

Results of the ANCOVA of the log percent thiamin loss in the various species were compared (Table 1). To make the rate of loss data directly comparable considering variation in initial concentrations of thiamin, the data were converted to percent loss. Since the thiamin/hydroxyl radical reaction is first order with respect to thiamin, the percent loss figures were converted to log values to provide a linear plot. Note from those data in the column "Pr > F" the only significant factors were the dose (D) and intercept (initial values) due to various species (SPECIES). The effect of species on loss of thiamin (D*SPECIES) was significant. Upon comparison of the rate constants we found that this loss in beef was different from that in pork, lamb and turkey leg, but not turkey breast. None of the others was significantly different. The log [thiamin] regressions were initially calculated with dose × dose and dose × dose × species terms. Neither term was significant, that is, there was no curvature in the log plot, hence the terms were not included (Table 1).

Reductants

The reducing capacity of muscle tissues affects the rate of thiamin loss on gamma irradiation (Fox et al., 1993). In that study the range of values was wide enough to calculate the dependence of the rate of loss on reducing capacity. In our current study the reducing capacities of the tissues were clustered in a range of 16 to 20 mM reductant, which was not broad enough to calculate a significant slope. Irradiated samples were also titrated with DCPIP, but the effect of radiation dose on reducing capacity was not significant.

Riboflavin

A net loss of riboflavin occurred in 9 of the 15 samples, but individually none of the rate constants was significant (Table 2). These data were subjected to an ANCOVA and collectively the loss was significant at the Pr > F level of 0.019. An analysis of the slopes indicated no difference in slopes due to species (Pr > | T | values all >0.05). We previously reported an increase in the amount of riboflavin measured in meats with a maximum at ca. 4 kGy (Fox et al., 1989), but we did not observe an increase in the current study, although there was no change up to about 3 kGy.

Sulfhydryl

One of the pork samples showed a decrease with radiation dose in sulfhydryl by Ellman's reagent, and one of the beef samples showed an increase. The remainder of the samples showed no significant changes. An ANCOVA showed no significant variation.

pH

Thiamin is stable to oxidation in acid solutions (Gubler, 1984) and a difference in pH could affect the rate of its loss. One pork sample had a low pH of 5.07 and one turkey leg had a high pH of 6.42. The average was 5.68 ± 0.28, within the range for post-rigor meats, but there no significant difference occurred in the pH of various species. Hence we could not verify whether pH had any effect on rate of thiamin loss.

Proximate analysis

Data for proximate analyses of the various species were compared (Table 3). The values for beef were all different from the highest or lowest values of the other species, primarily due to

Table 3—Proximate analyses of beef, lamb, pork and turkey leg and breast

Component	Component percent				
	Beef (rank)	Lamb	Pork	Tleg[c]	Tbre[c]
Protein	19.42[b] (low)	20.05[a,b]	20.73[a,b]	20.12[a,b]	23.45[a]
Fat	5.20[a] (high)	3.85[a,b]	3.08[a,b]	2.65[a,b]	1.38[b]
Moisture	70.81[b] (low)	72.69[a,b]	72.00[a,b]	74.57[a]	72.65[a,b]
Ash	0.84[b] (low)	0.96[a,b]	1.08[a]	0.97[a,b]	1.03[a,b]

[a,b] Means in the same row with no superscript in common are significantly different by ANCOVA.

[c] Tbre, turkey breast; Tleg, turkey leg.

Table 4—Comparison of thiamin losses from two separate studies

	Reducing capacity[c]		Chemiclearance[d]	
	[R]	kGy^{-1}	[R]	kGy^{-1}
Average	23.2[a]	0.051[b]	19.1[a]	0.100[b]
Standard deviation	7.7	0.025	4.5	0.048
Range	13.4–42.6	0.013–0.107	9.3–29.4	0.021–0.205

Regression[e]	Reducing capacity	Chemiclearance
k_0	0.557	0.153
k_r	0.462	0.0285
r	0.57 ($p < 0.0001$ @ 15 df)	N.S.

[a,b] Data with same superscripts are not significantly different from each other.

[c] Fox *et al.*, 1993.

[d] This study.

[e] For equation 1.

its higher fat content. Values for protein and water content were correspondingly lower. At the other extreme, turkey breast had a low fat content and a high protein content as expected for white muscle tissue. The extreme values in the ranges for each component were significantly different from each other, but neither extreme was significantly different from values in between. There was no correlation between proximate analysis values and losses of thiamin or riboflavin.

DISCUSSION

Vitamin loss

We compared our results with previously published values. Results for thiamin loss in beef, lamb, pork and turkey as related to the reducing capacity of the tissues (this study) were compared (Table 4) with a similar report of thiamin loss in beef, pork and chicken skeletal muscle and liver (Fox et al., 1993). Averages of the reducing capacity or the rates of thiamin losses were not significantly different, that is, the general ranges and scatter were the same. As noted previously (Fox et al., 1993), the reducing capacity is not the only factor controlling rates of thiamin loss, which probably explains the higher variation in rate constants as compared with reducing capacities. The regressions of the rate constants on the reducing capacities were calculated. The regression of the first set of data was significant but the regression for this study was not. The data in this study were clustered about 19–20 mM reducing capacities, and the range was too narrow to calculate a significant regression.

In a study of thiamin determination in matched chicken breasts (Fox et al., 1992) the coefficient of variation due to individual birds was ±15%, so that any variation beyond that range could be considered to be due to the treatment. In a study of loss of thiamin and other vitamins in pork chops and chicken breasts (Fox et al., 1989) loss of thiamin in pork was about three times that of chicken. However, that study was designed to determine vitamin loss in meats as processed and the chops and breasts were not under the same atmospheres or conditions. Ground meat was used for a study of vitamin loss as related to reducing capacity (Fox et al., 1993), whereas the meats were powdered in our current study, yet results were the same. Reducing capacities showed a 3-fold range in both studies (Table 4) and there was an 8-fold range of rate constants in the first study and a 10-fold range in our current study. Thus, while the rate of loss of thiamin in beef was twice that of lamb and was significant in view of an expected range of at least ten-fold, a two-fold variation is not particularly notable from a practical viewpoint.

Thiamin loss as a function of other tissue components

Of the several species, only beef was consistently different with a higher rate of thiamin loss. Beef also had higher fat and lower protein and moisture contents than the other meats. However, when beef was compared with the other meats, no correlation was found between thiamin loss and any of the tissue components, including water. In a study of the rehydration of freeze-dried pork (Fox et al., 1994), the rate of thiamin loss negatively correlated with water content, but the range of water contents was much greater. Lamb showed the lowest rate of thiamin loss, but none of the proximate analyses values differed between beef and lamb. As noted earlier, no correlation was found between thiamin loss and the reducing capacity of the several tissues we studied here. We concluded that the ranges of values were too low with respect to the range of thiamin loss values to establish significant relationships.

Vitamin loss in meats

Meats, in general, are not a major source of either B vitamin, since they contain about 1 μg thiamin/g and 2 μg riboflavin/g. Pork is an exception and contains ~ 10 μg thiamin/g (HNIS, 1987). Since first-order rate constants are independent of concentration (kGy^{-1}), the percent loss figures per given dose were directly comparable between all samples. At an average rate constant of 0.100 kGy^{-1}, the loss at 2 kGy would be 18%, but, based on the extremes of the ranges of the two studies reported, (0.0131 and 0.205 kGy^{-1}, Table 4), thiamin losses as low as 3% and as high as 34% could be expected. This amount of loss in all meats, except pork, would have little effect on the dietary intake of thiamin in the average consumer population. The major sources of thiamin and riboflavin, as well as the other B vitamins, are grain products, especially enriched flours and breads, in which the concentration of thiamin is about 8 μg/g and riboflavin 5 μg/g. From the data of Block et al. (1985) pork was calculated to supply about 9.00% of the thiamin in the American diet (Fox et al., 1989). At the average levels of thiamin losses, we found the loss in the American diet due to irradiation of pork to 3 kGy can be calculated as 2.3%, assuming that all pork and pork products were irradiated. The losses to the diet from irradiation of all other meats would be one-tenth of this value. Any detriment from such slight losses would seem to be more than compensated by the advantage of controlling bacteriological contamination through irradiation processing.

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Lipid Stability of Beef Model Systems with Heating and Iron Fractions

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ABSTRACT

Catalytic effects of different temperatures (55, 70, 85, and 100°C) on lipid oxidation were studied in aqueous- and chloroform/methanol-extracted beef model lipid systems containing iron forms inherent in beef (water-extractable, diffusate, nondiffusate, ferritin, myoglobin, hemoglobin), hematin, $FeCl_2$, or $FeCl_3$. Heating increased thiobarbituric acid and peroxide values in both systems. All forms of iron catalyzed lipid oxidation in aqueous systems, with greatest oxidation by heme and low molecular weight iron fractions. Oxidation in lipid extracts was not increased by ferritin, $FeCl_2$, or $FeCl_3$, but heme iron was the major oxidation catalyst. Lipid stability decreased with addition of any iron forms inherent in beef or with increased heating, which helps understanding of rapid oxidation of meat during refrigerated storage or after cooking.

Key Words: beef, lipid stability, iron fractions, heme, heating

INTRODUCTION

LIPID INSTABILITY LEADS to quality deterioration in stored meat and meat products. Cooked meat developed rancid flavor more rapidly than uncooked meat during refrigerated storage, resulting in "warmed-over flavor" (WOF) (Tims and Watts, 1958). Extent of lipid oxidation was affected by heating temperature (Keller and Kinsella, 1973; Pearson et al., 1977; Huang and Greene, 1978; Lillard, 1987; Arganosa et al., 1989; Tanchotikul et al., 1989).

Many different iron complexes, including low molecular weight compounds, heme compounds, and storage forms such as ferritin and hemosiderin have been found in meat (Hazell, 1982; Stryer, 1988). Heme pigments, myoglobin (Mb) and hemoglobin (Hb), have generally been considered major catalysts of lipid oxidation in red meat, especially when uncooked (Younathan and Watts, 1959; Tappel, 1962; Greene, 1971; Love, 1983, 1987; Rhee, 1988). Nonheme iron accelerated lipid oxidation in cooked meat (Sato and Hegarty, 1971; Love and Pearson, 1974; Igene et al., 1979). Heme and nonheme iron catalyzed oxidation in both raw and cooked meat systems (Wills, 1966; Liu and Watts, 1970), but specific forms of iron that catalyze lipid oxidation in meat have not been well defined. Distribution of various iron fractions could be altered by heat (Schricker et al., 1982; Chen et al., 1984; Han et al., 1993), which would affect lipid oxidation in cooked meat. The various forms of iron in meat impair determination of the effects of each form in lipid oxidation. Our objective was to compare thermal changes in aqueous- or chloroform/methanol-extracted beef lipid systems that contained no iron, with specific forms of iron fractions in amounts inherent in beef, to determine effects on catalysis of lipid peroxidation.

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MATERIALS & METHODS

Meat source and preparation

Bovine M. longissimus thoracis muscle (≈300 g) (pH 5.6) from each of four beef carcasses from grain finished market weight steers at the LSU Agricultural Center Meat Laboratory were vacuum-packaged individually in polyethylene/vinyl pouches (Koch Supplies, Kansas City, MO, oxygen and water transmission rates of 9.3 cc/m²/24 hr at 0°C) and stored at −18°C until analysis (≈2½ mo). Moisture and fat in beef samples were determined by rapid microwave procedures (AVP80 and Automatic Extraction System, CEM Corporation, Matthews, NC). Beef samples (≈300 g) were thawed at room temperature (27°C) for 2 hr, trimmed of visible fat and connective tissue, and cut into small pieces (<1 cm cubes) with a knife. All samples were mixed and ground through a 4.7 mm stainless steel plate in a grinder with stainless steel parts prior to preparation of model systems. A control sample (≈100g) was randomly selected from the ground beef, vacuum-packaged in polyethylene/vinyl pouches, and stored at −18°C for comparison of lipid stability with model systems.

Aqueous muscle tissue extraction

An aqueous residue model system similar to that of Sato and Hegarty (1971) was prepared by homogenizing samples (≈1000 g) with deionized water (1 L) (Waring blendor, Hartford, CT) at low speed for 2 min at room temperature (27°C) before centrifugation (3000 × g, 20 min at 4°C). Supernatants were combined from four repeated extractions, and later fractionated. Residues from each extraction were re-homogenized with 1L deionized water before re-centrifugation to provide a nearly colorless insoluble residue. Water-extracted residues (100g) were vacuum-packaged in polyethylene/vinyl pouches and frozen for −18°C storage as the aqueous residue model systems until analysis (2 wks). Total iron in wet-ashed samples was measured by atomic absorption spectrophotometry (AAS) (Schricker et al., 1982; Guzman, 1987).

Organic solvent extraction

Beef intramuscular fat was extracted with chloroform and methanol (2:1 v:v) from the water-extracted meat residues by the method of Folch et al. (1957). Chloroform was evaporated by vacuum drying. Extracted fat was dissolved in absolute alcohol (40.5% lipid by weight) before mixing with 0.025M phosphate buffer, pH 6.0 (1:9 v:v) at low speed for 30 sec (Wills, 1965). Lipid system extracts corresponding to original aqueous extracts were prepared immediately before each use.

Aqueous muscle component fractionation

Supernatants from aqueous residues were combined, freeze-dried (Consol 24 freeze drier, Virtis, Gardiner, NY), and fractionated into ferritin, Hb, and Mb fractions using procedures of Hazell (1982). Ten mL samples (of 3g freeze-dried extract in 15 mL deionized water) were loaded onto a 100 × 2.5 cm column (Bio-Rad, Richmond, CA) containing Ultrogel AcA 34 (Spectrum, Los Angeles, CA) and eluted with 0.2M potassium phosphate buffer (pH 6.8) at 0.42 mL/min. Twelve hours after sample loading, fractions were collected at 10-min intervals for 90 min. Bovine Mb (purified from beef, Yamazaki et al., 1964), bovine Hb (Sigma Chemical Co., St. Louis, MO) and ferritin from horse spleen (Sigma Chemical Co., St. Louis, MO) were used as standards to identify ferritin, Hb, and Mb. Fractions were pooled, freeze-dried, and stored in a desiccator at 4°C.

Diffusate and nondiffusate iron fractions were prepared by dissolving freeze-dried aqueous extract (3 g) in deionized water (15 mL). Each solution (10 mL) was dialyzed against deionized water (60 mL) at 4°C for 48 hr with dialysis tubing (4.5 cm, 12,000 dalton molecular weight cutoff, Fisher Scientific, Pittsburgh, PA). Diffusate and non-diffusate

Table 1—Concentrations of iron added to beef model systems

Iron type	Concentration
Water extractable iron	17.0 ppm
Diffusate iron	1.75 ppm
Non-diffusate iron	15.3 ppm
Hemoglobin	0.8 mg/g
Myoglobin	3.5 mg/g
Ferritin	0.4 ppm
Hematin	2.7 ppm
$FeCl_2$	1.75 ppm
$FeCl_3$	1.75 ppm

fractions were freeze-dried and stored in a desiccator at 4°C before determination of iron by AAS (Han et al., 1993).

Iron addition to model systems

Each of nine forms of iron (ferrous chloride, ferric chloride, and hematin from Sigma Chemical Co., St. Louis, MO; ferritin, Hb, Mb, water-soluble, diffusate, and nondiffusate fractions) were added independently into each model system at concentrations (Table 1) which corresponded to mean values of iron for each component in raw beef samples (Han et al., 1993). Ferrous chloride and ferric chloride equivalent to the diffusate iron were added to provide a relative determination of catalysis effects due to oxidative state (ferrous or ferric) because exact concentrations of free Fe^{2+} and Fe^{3+} in bovine muscle are unknown. Required amounts of each iron fraction were accurately weighed (Mettler AT 261 Balance, Greifensee, Switzerland) and mixed with model system (100g) for 30 sec before division of the model system into five portions (≈20 g) in 50-mL Erlenmeyer flasks.

Model system heating

Four flasks with the same model system and iron forms were placed in 250-mL beakers in a tightly covered steam water bath at 101.6° ± 0.5°C (Precision Scientific Co., Chicago, IL). Thermocouples that extended through rubber stoppers in the flasks were linked to a Digital Data Acquisition System (MackMac 1240-Touch Display, GreenSpring Computers, Menlo Park, CA). One flask was removed at random when internal temperatures reached 55, 70, 85, or 100°C. Flasks were cooled in ice water for 30 min and stored at 4°C for 48 hr before assessment of lipid stability. Ground bovine muscle and corresponding model systems with no added iron served as controls and were heated and stored under conditions similar to treatment samples.

Lipid oxidation and fatty acid composition

Oxidative stability was evaluated by measuring 2-thiobarbituric acid reactive substances (TBARS) (Tarladgis et al., 1960) and peroxide value (PV) (Koniecko, 1985) for each of two samples from each treatment combination. Lipids were extracted from model systems (Folch et al., 1957). Fatty acid methyl esters from model systems were prepared (Metcalfe et al., 1966) and quantified by gas chromatography at the USDA-ARS Southern Regional Research Center Food Quality Laboratory, New Orleans, LA. Samples were injected onto a fused silica column (0.32 × 30 mm, Supelco SP2340) with 1 mL/min nitrogen carrier gas flow, injector 220°C and detector 250°C. Oven temperatures were increased at 10°C/min from 120 to 150°C, 3°C/min to 200°C and 5°C/min to 220°C.

Statistical analysis

The experiment was replicated two times as a split-plot design with type of iron as whole plot and temperature as sub-plot. Analyses of variance (SAS Institute, Inc., 1985) with main effects of temperature and type of iron added were performed for dependent variables of PV or TBARS. Least-squares means were compared with t-tests when analyses of variance indicated treatment effects ($P < 0.05$). Pearson correlations were performed to determine relationships between PV and TBARS values.

RESULTS & DISCUSSION

Model system composition

Iron content in the aqueous beef residue was 8.0 ppm, similar to our previous results in corresponding bovine longissimus muscle (Han et al., 1993). Trace amounts of iron in lipid extract system (0.08 ppm) could have been from hemoproteins not removed during aqueous extraction, from intramuscular fat, or due to phosphate buffer. In preparing the aqueous residue model system, water had been used to remove most pigments, water-extractable iron, and minerals from meat sample (Sato and Hegarty, 1971; Chen et al., 1984) so the residue contained water-insoluble iron, water-insoluble proteins, lipids, and muscle fiber. Average times to reach internal temperatures of 55, 70, 85, and 100°C were 2.5, 4.5, 6.7 and 20.8 min for aqueous residues and 1.8, 3.5, 5.2, and 12.0 min for lipid extracts (determined in two replications and another independent test). Moisture and fat of the aqueous residue model system were 66.29 ± 1.2% and 2.46 ± 0.2%, respectively. Fat content of the intramuscular lipid system (lipid extract) was 4.05%, near the fat content in raw bovine longissimus muscle.

Table 2—Percentage composition[a] of fatty acids in beef model systems

Name	Fatty acid	Aqueous residue	Lipid extract
Myristic	14:0	1.83[c]	3.81[d]
Pentadecanoic	15:0	0.41[c]	2.05[c]
Palmitic	16:0	22.10[c]	23.86[c]
Palmitoleic	16:1	3.31[c]	10.42[c]
Margaric	17:0	1.93[c]	5.27[d]
Stearic	18:0	21.23[c]	18.56[d]
Oleic	18:1	46.83[c]	35.44[d]
Linoleic	18:2	1.60[c]	1.43[c]
Linolenic	18:3	0.21	ND
Arachidic	20:0	0.15	ND
Gadoleic	20:1	0.49[c]	4.12[d]
Heneicosanoic	21:0	0.56[c]	1.39[c]
Total SFA[b]		48.18[c]	52.19[d]
Total UFA[b]		52.27[c]	47.81[d]
Total PUFA[b]		1.64[c]	1.42[c]
Total lipid, mg/g		24.6	40.5

[a] Least squares means of five repeated measurements from the same sample.
[b] SFA = saturated fatty acids; UFA = unsaturated fatty acids; PUFA = polyunsaturated fatty acids.
[cd] Means in same row with same superscripts are not different ($p>0.05$).
ND = not detected.

Fatty acid composition (Table 2) showed the aqueous system had a higher proportion of oleate and total unsaturated fatty acids than the lipid system. Predominant saturated fatty acids were palmitic and stearic, and the predominant unsaturated fatty acid was oleic. Linoleic was the polyunsaturated fatty acid in the greatest amount. The fatty acid composition of the model systems was similar to the composition reported for intact longissimus muscle (Westerling and Hedrick, 1979; Bodwell and Anderson, 1986; Sweeten et al., 1990).

Lipid stability in aqueous residue system

Peroxide value measures the primary peroxide products (Koniecko, 1985) during initiation of lipid oxidation while TBARS measure secondary products, including malondialdehyde (Tarladgis et al., 1960; Fennema, 1985). Analyses of variance (not shown in tables) indicated that lipid oxidation in the aqueous system increased with heating, type of iron added, and their interaction. Higher ($p < 0.05$) PV and TBARS were caused by heating of control and treated systems (Tables 3 and 4). However, lipid oxidation in the control with no iron added was less with heating to 100°C than in treatment samples.

Lipid stability of ground beef control was also decreased by heating (Tables 3 and 4). PV and TBARS values of beef residue with water-soluble iron were similar to those obtained from ground longissimus muscle. These findings indicated that water-soluble fractions were major catalysts of lipid oxidation and water-insoluble iron had limited catalytic activity. This confirmed Apte and Morrissey (1987b), who had reported that the water-extracted muscle system containing added total soluble iron gave TBARS values similar to those obtained for intact muscle systems. Torrance et al. (1968) and Hazell (1982) indicated that water-soluble iron fractions included diffusate iron, hemoprotein iron, and ferritin iron.

Table 3—Peroxide values[a] in aqueous beef residue model systems with heating and iron

Iron treatment	Temperature of heating, °C				
	Unheated	55	70	85	100
Control (no added iron)	2.64^{c}	4.69^{c}	9.46^{f}	11.01^{f}	10.75^{f}
Water-soluble iron	9.64^{ef}	12.79^{h}	16.88^{j}	19.72^{l}	19.22^{l}
Diffusate iron	8.72^{e}	11.42^{gh}	15.18^{ij}	18.25^{kl}	17.80^{kl}
Non-diffusate iron	8.68^{e}	10.79^{g}	15.01^{ij}	17.61^{k}	16.96^{k}
Hemoglobin	7.84^{e}	9.96^{fg}	14.17^{hi}	17.08^{k}	16.70^{ik}
Myoglobin	7.79^{e}	10.15^{fg}	14.21^{hi}	17.00^{k}	16.69^{ik}
Ferritin	4.25^{cd}	8.78^{f}	13.09^{gh}	15.01^{i}	16.35^{ik}
Hematin	5.36^{d}	10.31^{fg}	12.01^{g}	14.92^{i}	15.06^{i}
$FeCl_2$	11.38^{f}	14.56^{h}	20.75^{l}	26.13^{m}	26.92^{m}
$FeCl_3$	4.63^{cd}	10.29^{fg}	16.05^{j}	19.64^{l}	19.73^{l}
Ground beef control[b]	9.99	13.63	18.60	20.68	19.92

[a] Least squares means (milliequivalents peroxide/kg sample) of 4 measurements, s.e.m. = 0.83.

[b] Ground longissimus muscle, s.e.m. = 0.51.

Means in same row or column with same superscripts are not different (p>0.05).

Table 4—TBARS values[a] in aqueous beef residue model systems with heating and iron

Iron treatment	Temperature of heating, °C				
	Unheated	55	70	85	100
Control (no added iron)	0.58^{c}	0.92^{d}	1.28^{e}	1.56^{f}	1.50^{f}
Water-soluble iron	2.66^{k}	2.93^{m}	3.59^{s}	4.05^{u}	3.90^{t}
Diffusate iron	2.14^{i}	2.57^{j}	2.98^{n}	3.47^{r}	3.36^{q}
Non-diffusate iron	1.98^{h}	2.14^{i}	2.62^{k}	3.19^{p}	3.10^{o}
Hemoglobin	1.64^{g}	1.93^{h}	2.52^{j}	3.13^{op}	3.04^{n}
Myoglobin	1.60^{g}	1.90^{h}	2.58^{jk}	3.08^{o}	3.01^{n}
Ferritin	0.83^{e}	1.60^{g}	2.12^{i}	2.78^{l}	3.09^{o}
Hematin	1.13^{f}	1.57^{g}	1.93^{h}	2.80^{l}	2.88^{m}
$FeCl_2$	4.32^{v}	4.87^{w}	6.01^{x}	6.59^{y}	6.99^{z}
$FeCl_3$	0.75^{d}	2.64^{k}	3.50^{r}	3.85^{t}	3.86^{t}
Ground beef control[b]	2.83	3.15	3.86	4.28	4.01

[a] Least squares means (mg malondialdehyde/kg sample) of 4 measurements, s.e.m. = 0.02.

[b] Ground longissimus muscle, s.e.m. = 0.01.

Means in same row or column with same superscripts are not different (p>0.05).

Similar results were obtained with either PV or TBARS to assess effects of iron treatment on lipid oxidation in aqueous systems. The Pearsons correlation coefficient between the two was 0.84, which indicated that iron treatments had similar effects on formation of primary and secondary oxidation products in the aqueous systems.

Iron treatment caused higher ($p < 0.05$) PV in the unheated system for all components except ferritin and $FeCl_3$ (Table 3). The TBARS in the unheated system were higher ($p < 0.05$) for all iron treatments than for controls (Table 4). Heating to 55°C and 70°C increased ($p < 0.05$) PV and TBARS for all iron treatments. Further increases ($p < 0.05$) in PV were observed with heating to 85°C for samples treated with water-soluble iron, hematin, Hb, Mb, and both $FeCl_2$ and $FeCl_3$ but no further increases were obtained by heating to 100°C. TBARS were higher ($p < 0.05$) at 85°C for all treatments and were the highest ($p < 0.05$) at 100°C for hematin, ferritin, $FeCl_2$, and $FeCl_3$. TBARS declined ($p < 0.05$) after heating to 100°C in water-soluble, diffusate iron, non-diffusate iron, Hb, and Mb aqueous treatments.

Heat might have induced changes in microsomes, mitochondria, and membranes, causing them to be more susceptible to oxidation (Cross et al., 1987). Heat might also disrupt muscle membranes and result in exposure of more labile lipid components to oxygen and other reaction catalysts (Sato and Hegarty, 1971). However, the Maillard reaction between amino acids or proteins and carbohydrates could proceed in meat at high temperatures (Bailey et al., 1987). Maillard reaction products (MRP), such as melanoidins and reductone precursors had strong antioxidant properties in cooked meats (Hamm, 1966; Sato et al., 1973; Huang and Greene, 1978; Bailey et al., 1987). According to Hamm (1966), Maillard reactions in meat began at about 90°C and increased with temperature and heating time. Therefore, lipid oxidation at 100°C in meat residues was dependent on two opposing factors, iron and heat catalysis and MRP inhibition. Catalytic potential of ferritin, Fe^{2+}, or Fe^{3+} might exceed antioxidant effects of MRP, which would result in a slight increase in lipid oxidation in aqueous residues with those components at 100°C.

The catalytic potential of Fe^{3+} and ferritin in raw beef residues was low until samples were heated. Johns et al. (1989) speculated that ferrous ions were rapidly oxidized to ferric form. Sato and Hegarty (1971) had indicated that a balance of ferrous and ferric ions was necessary for rapid oxidation. Apte and Morrissey (1987a) reported that intact ferritin was not a catalyst of lipid oxidation in uncooked meat. Heat could cause release of free iron from ferritin which then catalyzed lipid oxidation (Apte and Morrissey, 1987a; Decker and Welch, 1990). Sato and Hegarty (1971) also reported that ferric chloride did not increase lipid oxidation compared with ferrous chloride. Results of Harel and Kanner (1985a,b) suggested that ferric heme pigments may be effective catalysts only in presence of hydrogen peroxide.

Lipid oxidation in meat residues catalyzed by Hb fraction, Mb fraction, or hematin increased ($p < 0.05$) as temperature increased with the largest increase of PV and TBARS during heating from 55 to 85°C (Tables 3 and 4). Increased hemoprotein denaturation and heme iron reduction occur within this temperature range (Han et al., 1993). Increased lipid oxidation by hemoprotein after heat treatment might be due to conformation changes of hemoproteins, which results in greater exposure of catalytic heme group to unsaturated fatty acids (Greene and Price, 1975; Igene et al., 1979). It could also result from release of nonheme iron from heme (Chen et al., 1984).

Catalytic activity of Mb and Hb fractions were similar, while hematin caused less catalysis in model systems, perhaps due to its lower concentration. Both Mb and Hb are composed of heme moiety (iron atom surrounded by porphyrin ring) and polypeptide chains of amino acids (Ladikos and Wedzicha, 1988). In the native form, each molecule of Hb has four polypeptide chains that link with heme moieties. A molecule of Mb has only one polypeptide chain and one heme moiety (Dickerson and Geis, 1983). The concentration of Mb in model systems was four times that of Hb which accounted for the similar catalytic effects. Heme iron did not accelerate lipid oxidation when Hb at 10 to 25 mg/g and Mb at 5 mg/g were added to water-extracted muscle residues (Sato and Hegarty, 1971). The high concentration of heme pigments in that study may have functioned as oxidation inhibitors (Fox and Benedict, 1987). Johns et al. (1989) stated that oxygenated forms of heme pigments catalyzed lipid oxidation while metmyoglobin or methemoglobin pigment forms had low catalytic activity.

$FeCl_2$ had the highest PV and TBARS among all forms of iron, which suggested that $FeCl_2$ had the strongest catalytic effects in lipid oxidation in both raw and cooked beef residue. However, PV or TBARS in the beef residue catalyzed by diffusate iron were much lower than those catalyzed by Fe^{2+}. An explanation might be the presence of oxidation inhibitors (e.g., glutathione, cysteine, and histidine) in the diffusate fraction (Sato and Hegarty, 1971; Decker and Schanus, 1986). Although oxidation inhibitors may have been present, lipid oxidation (TBARS values) in the beef residues catalyzed by diffusate iron was the same or higher than that catalyzed by non-diffusate iron at the same heating temperature. This indicated that iron in the low molecular weight fraction (diffusate iron) might have a greater catalytic effect. However, heme iron exerted major catalytic effects in meat residues.

PV or TBARS values catalyzed by non-diffusate iron, which included ferritin, Hb and Mb, were much lower than the sum total value when the PV or TBARS of individual ferritin, Hb, and Mb fractions were added. The total PV or TBARS in aqueous residues catalyzed by adding effects of diffusate and non-diffusate iron were also not equal to those catalyzed by water extractable iron, suggesting that the catalytic effects of iron were not additive. Determination of the relative contribution of dif-

Table 5—Peroxide values[a] in beef intramuscular lipid model systems with heating and iron

Iron treatment	Temperature of heating, °C				
	Unheated	55	70	85	100
Control (no added iron)	2.51^{b}	4.84^{c}	8.15^{f}	9.70^{fg}	10.65^{g}
Water-soluble iron	6.21^{d}	9.12^{f}	12.42^{hi}	13.80^{ij}	14.24^{j}
Diffusate iron	5.10^{cd}	6.93^{e}	9.41^{fg}	10.59^{g}	10.86^{g}
Non-diffusate iron	6.14^{d}	8.50^{f}	12.25^{hi}	13.35^{i}	13.52^{ij}
Hemoglobin	5.70^{cd}	7.90^{e}	11.73^{h}	13.14^{hi}	13.47^{ij}
Myoglobin	5.61^{cd}	7.78^{e}	11.73^{h}	13.11^{hi}	13.32^{ij}
Ferritin	2.64^{b}	4.86^{c}	8.21^{f}	9.77^{g}	10.60^{g}
Hematin	4.57^{c}	7.06^{e}	10.04^{g}	11.07^{g}	11.43^{g}
$FeCl_2$	3.08^{b}	4.98^{c}	8.31^{f}	9.84^{fg}	10.98^{g}
$FeCl_3$	2.92^{b}	4.99^{c}	8.30^{f}	9.90^{g}	10.99^{g}

[a] Least squares means (milliequivalents peroxide/kg sample) of 4 measurements, s.e.m. = 0.68. Means in same row or column with same superscripts are not different ($p>0.05$).

Table 6—TBARS[a] in beef intramuscular lipid model systems with heating and iron

Iron treatment	Temperature of heating, °C				
	Unheated	55	70	85	100
Control (no added iron)	0.84^{b}	0.94^{c}	1.23^{e}	1.33^{f}	1.40^{fgh}
Water-soluble iron	1.56^{i}	1.75^{j}	2.06^{l}	2.34^{m}	2.43^{n}
Diffusate iron	0.93^{c}	1.05^{d}	1.32^{g}	1.43^{h}	1.46^{h}
Non-diffusate iron	1.45^{h}	1.66^{i}	1.98^{k}	2.31^{m}	2.37^{mn}
Hemoglobin	1.32^{f}	1.48^{h}	1.86^{j}	2.19^{l}	2.22^{l}
Myoglobin	1.32^{f}	1.45^{h}	1.88^{j}	2.20^{l}	2.29^{l}
Ferritin	0.85^{b}	0.94^{c}	1.24^{e}	1.33^{f}	1.40^{fgh}
Hematin	1.08^{d}	1.24^{e}	1.50^{h}	1.60^{i}	1.69^{i}
$FeCl_2$	0.83^{b}	0.95^{c}	1.25^{e}	1.34^{fg}	1.40^{fgh}
$FeCl_3$	0.84^{b}	0.93^{c}	1.23^{e}	1.33^{f}	1.39^{fgh}

[a] Least squares means (mg malondialdehyde/kg sample) of 4 measurements, s.e.m. = 0.03. Means in same row or column with same superscripts are not different ($p>0.05$).

ferent fractions of iron to lipid oxidation was impossible because of non-additive catalysis and possible oxidation inhibitors.

Lipid stability in intramuscular lipid extracts

Lipid was extracted from aqueous residues by a mixture of chloroform and methanol to study the catalytic effects of iron fractions on lipid oxidation in the absence of water-insoluble proteins and muscle fibers. Animal intramuscular fat cells and the lipid in muscle membranes are in close proximity to lean tissue and thus would probably be more important in lipid oxidation than other adipose tissues (Cross et al., 1987).

PV (Table 5) and TBARS (Table 6) for effects of heat and various forms of iron on lipid oxidation in lipid extracts were compared. Analyses of variance indicated that type of iron and heat affected ($p < 0.05$) lipid oxidation in lipid extracts. Interactions ($p < 0.05$) existed between heating temperature and type of iron catalyst for TBARS indicating that catalytic effects of various forms of iron differed due to heating temperatures. However, analyses of variance for PV showed no interactions ($p > 0.05$) between heating temperatures and types of iron. Possible explanations for this difference between PV and TBARS might be that the PV method was less accurate, with larger standard errors. Also secondary lipid oxidation may be influenced by factors other than primary oxidation.

The Pearson correlation coefficient between PV and TBARS was 0.79. Lipid stability in unheated lipid extract control with no added iron was higher ($p < 0.05$) than in heated and/or iron treated extracts, with exceptions of ferritin, $FeCl_2$, and $FeCl_3$ in unheated treatments.

Lipid oxidation in the lipid model system increased ($p < 0.05$) at 55°C and 70°C for most treatments but did not increase much more with further heating as indicated by PV (Table 5). Water-soluble iron, nondiffusate iron, Hb, and Mb were the most active ($p < 0.05$) catalysts, which may be related to the catalytic effects of denaturation in heme proteins (Love, 1983; Han et al., 1993). Diffusate iron had little catalytic effect and ferritin, Fe^{2+}, and Fe^{3+} did not have any effects on lipid oxidation compared with controls. Chelation of free iron by phosphate, and/or formation of insoluble salts between free iron (Fe^{2+} or Fe^{3+}) and phosphate might weaken the catalytic effects by diffusate iron forms and ferritin (Han, 1992). Water-extractable iron fractions included fractions of diffusate and non-diffusate iron. The non-diffusate iron fraction contained mainly hemoproteins (Mb and Hb) and a small amount of ferritin. Both water-extractable and non-diffusate iron had strong catalytic effects on lipid oxidation indicating heme iron of the hemoprotein was probably responsible.

Similar degrees of lipid oxidation were found when iron catalysts were omitted from the two model systems. Addition of iron catalysts increased PV and TBARS in aqueous residues more than in lipid extracts. Kanner (1994) indicated that cytosol contains pro- and antioxidants that would affect *in situ* lipid peroxidation. Because the cytosolic components that might influence lipid peroxidation were not measured in our study, we may only hypothesize that interactions between compounds that remained in muscle residues and iron resulted in increased lipid oxidation in the aqueous extracts.

CONCLUSIONS

ALL FORMS OF IRON present in beef contributed to development of lipid oxidation. Measurement of catalytic effects of various forms of iron within the same study indicated the relative catalytic order on lipid oxidation in unheated beef was water-soluble iron > diffusate iron > nondiffusate iron > Hb ≈ Mb > hematin > ferritin > water-insoluble iron. In the intramuscular lipid system, ferrous and ferric chloride did not induce increases in lipid oxidation. Heating increased lipid oxidation, most particularly with the forms of iron inherent in beef, in proportion to increased heating. The order for oxidation catalysis in cooked meat was reversed for ferritin and hematin. Chelating agents for iron should probably be used to minimize oxidation of lipids in meat during refrigerated storage or after heating.

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Consumer Evaluation of Raw and Fried Chicken After Washing in Trisodium Phosphate or Lactic Acid/Sodium Benzoate Solutions

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ABSTRACT

Whole raw chickens (180) were washed in tapwater (control), 12% trisodium phosphate (TSP), or 0.5% lactic acid/0.05% sodium benzoate (LB). Consumer panelists evaluated raw, treated, whole chickens as well as fried breast and thigh pieces. TSP and LB solutions did not affect ($P < 0.05$) external color, texture, flavor, moistness, or overall acceptability of fried chicken or consumer purchase intent. Evaluation of raw chickens 90 min after treatment with LB or after 7 days storage at 1°C revealed that overall acceptability, color and purchase intent were lower compared to controls. Sensory quality of raw chickens was not adversely affected by TSP. We concluded that 12% TSP or 0.5% lactic acid/0.05% sodium benzoate solutions have good potential for dips to sanitize chickens intended for frying before presentation to consumers.

Key Words: chicken, trisodium phosphate, lactic acid, benzoic acid, consumer acceptance

INTRODUCTION

INCREASED CONSUMPTION OF POULTRY PRODUCTS in the U.S. has been accompanied by an increase in foodborne illnesses associated with poultry. Pathogenic bacteria which may be associated with poultry include *Salmonella* spp. (Bryan, 1980), *Campylobacter jejuni* (Stern et al., 1985), *Staphylococcus aureus* (Bergdoll, 1989), and *Listeria monocytogenes* (Brackett, 1988). Breeder facilities, feed ingredients, farms, processors, and distributors have been sources of *Salmonella* contamination (Shackelford, 1988). The potential for growth of psychrotrophic pathogens such as *L. monocytogenes* (Glass and Doyle, 1989) and *Yersinia enterocolitica* (de Boer et al., 1982) in refrigerated poultry products is of particular concern.

Researchers have investigated many techniques for decreasing microbial contamination of raw chicken carcasses. The use of chlorine and chlorine dioxide in chill water has been studied extensively (Lillard, 1979; Morrison and Fleet, 1985; Teotia and Miller, 1975). The efficacy of chlorine is diminished because of its reaction with organic compounds from poultry carcasses. Lillard (1979) reported that chill water containing chlorine dioxide reduced the number of viable bacteria on carcasses compared to carcasses chilled in untreated water. Thiessen et al. (1984) reported that skin color was bleached upon treatment with chlorine dioxide.

Sheldon and Brown (1986) reported that microbial populations on chicken carcasses treated with ozone and stored at 4.4°C were consistently lower than those on untreated carcasses. No changes in carcass skin color, lipid oxidation, or flavor were reported after ozone treatment. A reduction in incidence of *Salmonella* on chicken carcasses treated in chilled water containing lactic acid was reported by Morrison and Fleet (1985). However, lactic acid caused the skin of carcasses to appear bleached. Mulder (1984) reported that irradiation of poultry successfully reduced populations of *Salmonella* spp. However, irradiation treatment may also cause development of undesirable flavors and odors (Lamuka et al., 1992).

Treatment of poultry carcasses with trisodium phosphate (TSP) was effective in reducing populations of *Salmonella* and other pathogenic bacteria (Giese, 1993). Hollender et al. (1993) reported an increase in consumer acceptability of raw TSP-treated carcasses over untreated carcasses. Previous work from our laboratory (Hwang and Beuchat, 1994) indicated that washing poultry skin in a lactic acid/sodium benzoate solution resulted in reductions in populations of *Salmoneila, L. monocytogenes, C. jejuni*, and *S. aureus* compared to washing in water. Our objective was to determine if treatment of raw chickens with TSP and lactic acid/sodium benzoate solutions affected sensory quality of whole, raw carcasses and fried breasts and thighs.

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MATERIALS & METHODS

Sample preparation

Freshly slaughtered, whole raw chicken carcasses (1.5–1.6 kg/carcass) packed in crushed ice were obtained from Goldkist Farms (Carrollton, GA) and held at 1°C for 1 day before experimental treatments. Ten chickens were dipped in 75–80 L of chilled (1°C) tap water (control), 12% TSP (AvGARD™, Rhone-Poulenc Inc., Cranbury, NJ), or a 0.5% lactic acid and 0.05% sodium benzoate (LB) solution for 30 min, respectively. A dipping time of 15 sec in 12% TSP has been proposed by the USDA Food Safety and Inspection Service (Federal Register, 1994). The control and LB dipping times were consistent with the dipping time in chill water commonly used by chicken processors. Broilers were removed from the dip tank and allowed to drain at 22°C on a screen for 30 min. Five chickens from each group were randomly selected for evaluation of overall acceptability, color and purchase intent by a sensory panel. Breast and thigh pieces were cut from the other five chickens for studies designed to determine sensory quality of fried chicken.

Frying methods

Breast and thigh pieces of control and treated chickens were coated in unflavored batter and breading (Newly Weds Foods, Chicago, IL). Batches of 10 breasts or thighs were then fried in randomized order in peanut oil (Sessions Company Inc., Enterprise, AL) in a Henny Penny Pressure Fryer (Model 500, Henny Penny Corp., Eaton, OH). Each batch was fried 10 min at 177°C and 700 g/cm^2 of pressure. These conditions resulted in an internal temperature of 85°C. Fried breast and thigh pieces were kept warm in an oven (65–70°C) until served to panelists. The holding period did not exceed 1 h after frying.

Sensory evaluation

Panelists for evaluation of raw and fried chicken consisted of faculty, staff, and students from the University of Georgia Agricultural Experiment Station, Griffin, GA. Participants selected were those who purchased chicken at least two times each month and ate chicken at least once each week; all panelists ate breast and thigh meat. Three groups of 10 panelists each evaluated fried breasts and thighs on each of two separate days (total of 60 panelists). Different panelists were used on the two test days. Before each test, each panelist was asked to complete a demographic questionnaire for pertinent background information. This concerned age, sex, race, marital status, annual income, education, employment, eating frequency per day (including snacks), frequency of

Table 1—Mean scores for sensory characteristics of fried chicken[c]

Sensory characteristic	Piece	Mean score (n = 60) Control	TSP[d]	LB[d]
External color	thigh	6.4 ± 1.7[a]	6.8 ± 1.4[a]	6.3 ± 1.9[a]
	breast	6.3 ± 1.8[ab]	6.7 ± 1.6[a]	6.0 ± 1.8[b]
Texture	thigh	6.3 ± 1.6[a]	6.7 ± 1.5[a]	6.3 ± 1.5[a]
	breast	6.7 ± 1.4[a]	6.7 ± 1.6[a]	6.3 ± 1.8[a]
Flavor	thigh	5.8 ± 1.7[a]	6.3 ± 1.7[a]	5.7 ± 1.9[a]
	breast	6.3 ± 1.4[a]	6.5 ± 1.4[a]	6.0 ± 1.7[a]
Moistness	thigh	6.8 ± 1.3[a]	7.0 ± 1.3[a]	6.8 ± 1.2[a]
	breast	6.6 ± 1.6[a]	6.8 ± 1.5[a]	6.6 ± 1.4[a]
Overall	thigh	5.9 ± 1.7[a]	6.5 ± 1.7[a]	6.0 ± 1.6[a]
	breast	6.5 ± 1.4[a]	6.5 ± 1.6[a]	6.1 ± 1.7[a]

[a,b] Mean scores in the same row that are not followed by the same letter are significantly different ($P < 0.05$).
[c] A 9-point hedonic scale (9 = like extremely; 8 = like very much; 7 = like moderately; 6 = like slightly; 5 = neither like nor dislike; 4 = dislike slightly; 3 = dislike moderately; 2 = dislike very much; 1 = dislike extremely) was used.
[d] TSP = trisodium phosphate; LB = lactic acid/sodium benzoate solutions.

Table 2—Mean scores for surface appearance and purchase intent of whole raw chicken[c]

Sensory characteristic	Storage time (days)	Mean score Control	TSP[d]	LB[d]
Overall acceptability	0	6.1 ± 1.7[a]	5.6 ± 1.6[ab]	5.2 ± 1.6[b]
	7	5.8 ± 1.6[a]	5.7 ± 1.5[a]	4.8 ± 1.9[b]
Color	0	6.2 ± 1.7[a]	5.5 ± 1.6[b]	5.1 ± 1.6[b]
	7	5.9 ± 1.6[a]	5.8 ± 1.5[a]	4.6 ± 2.0[b]
Purchase intent	0	2.6 ± 0.9[a]	2.3 ± 0.9[ab]	2.2 ± 0.9[b]
	7	2.6 ± 0.7[a]	2.6 ± 0.7[a]	2.1 ± 0.8[b]

[a,b] Mean values in the same row that are not followed by the same letter are significantly different ($P < 0.05$).
[c] A 9-point hedonic scale (9 = like extremely; 8 = like very much; 7 = like moderately; 6 = like slightly; 5 = neither like nor dislike; 4 = dislike slightly; 3 = dislike moderately; 2 = dislike very much; 1 = dislike extremely) was used to evaluate acceptability and color. A 4-point scale (4 = more often than other raw chicken; 3 = same as other raw chicken; 2 = less than other raw chicken; 1 = never purchase) was used to determine purchase intent.
[d] TSP = trisodium phosphate; LB = lactic acid/sodium benzoate solutions

buying fried chicken, frequency of buying raw chicken, and preference for color of raw chicken.

Chicken pieces were fried in an area separate from the testing area to avoid cooking odors influencing the panelist ratings. Each panelist was served a fried split breast from the control and each of the treatment groups. The same procedure was used to evaluate fried thighs. The order of presentation of breasts and thighs was randomized. All samples were labeled with three digit random code numbers and served to panelists in random order within a 15-min timeframe. Samples were served monadically to panelists. Panelists were asked to score external color, texture, flavor, moistness, and overall acceptability using a 9-point hedonic scale (9 = like extremely; 8 = like very much; 7 = like moderately; 6 = like slightly, 5 = neither like nor dislike; 4 = dislike slightly; 3 = dislike moderately; 2 = dislike very much, 1 = dislike extremely). Panelists were asked to record their purchase intent for each piece on a four-point scale (4 = I would purchase more often than other fried chicken; 3 = I would purchase the same as other fried chicken; 2 = I would purchase less often than other fried chicken; 1 = I would never purchase).

After evaluating all six fried samples, panelists were asked to evaluate control and treated, raw, whole chickens in a separate test area. The time elapsed between dipping and evaluating was about 90 min. Five chickens from the control and each treatment were placed in groups on coded stainless steel trays. Panelists were asked to evaluate each group of chickens in randomized order. For each group of five chickens, panelists were asked to assign scores for overall acceptability and color on a 9-point hedonic scale (9 = like extremely; 8 = like very much; 7 = like moderately; 6 = like slightly, 5 = neither like nor dislike; 4 = dislike slightly; 3 = dislike moderately; 2 = dislike very much, 1 = dislike extremely). Additionally, panelists were asked to record purchase intent of each group on a 4-point scale (4 = I would purchase more often than other raw chicken; 3 = I would purchase the same as other raw chicken; 2 = I would purchase less often than other raw chicken; 1 = I would never purchase). Chickens were displayed at room temperature for no longer than 30 min before sealing them in plastic bags and storing at 1°C for 7 days. The same chickens were evaluated again by the same panelists on day 7 using the same evaluation protocol.

Statistical analysis

Values from sensory quality evaluation of fried and raw chicken were analyzed separately for significant differences ($P < 0.05$) using General Linear Model and Duncan's multiple range tests (SAS Institute, Cary. NC). Each value presented represents the mean of 60 values obtained from two replicate trials (six panel sessions). The results from the second trial were combined with those of the first trial after statistical analysis showed no significant differences between the two replications.

RESULTS & DISCUSSION

Demographic characteristics

Demographic information revealed that 57% of panelists were male and 43% were female, with ages 18 to 75 years. Their annual household income ranged from <$10,000 to >$70,000. The highest level of formal education ranged from junior high school to university post graduate degrees. Over 90% of panelists indicated that they buy raw chicken and eat fried chicken at least "1–2 times a month" but no more than "2–3 times a week." About 80% of the panelists were married or had previously been married. About 40% of the panelists preferred to purchase white raw chicken, 20% preferred to purchase yellow chicken, and the others had no color preference when purchasing raw chicken.

Sensory evaluation of fried chicken

With exception of external color of fried breast pieces, there were no significant differences between mean sensory characteristics of fried chicken prepared from either controls or chemically treated broilers (Table 1). Most scores fell between "like moderately (=7)" and "like slightly (=6)." External color of fried chicken breast prepared from chickens dipped in 12% TSP was rated significantly higher than that treated with LB. However, neither treatment resulted in significant change in external color compared to the control treatment.

No differences occurred between treatments with regard to consumer purchase intention for fried chicken. About 50% of the panelists indicated they would purchase the LB-treated fried chicken "as often" or "more often" than other fried chicken they currently purchase. These results indicate that, all other factors being the same, the use of LB solution as a dip would not change the purchase intent of consumers. Over 50% of the panelists indicated they would purchase TSP-treated chickens "as often" or "more often" than they would purchase other fried chicken. Results tend to confirm observations on the effect of TSP treatment on fried chicken reported by Hollender et al. (1993). Their research indicated that sensory qualities of fried chicken dipped in 8% TSP solution for 15 min was acceptable to consumers.

Evaluation of raw whole broilers

Differences ($p < 0.05$) in acceptability and color scores, and purchase intent for raw control and treated chickens were detected (Table 2). For all three characteristics, initially and after 7 days of storage at 1°C, LB-treated chickens were scored lower compared to controls. On day 7, panelists rated LB-treated chickens lower than TSP-treated chickens in all three categories. Still, over 30% of the panelists indicated they would purchase LB-treated chickens "as often" or "more often" than they would purchase other raw chickens. Hollender et al. (1993) indicated that panelists had purchase preferences significantly higher for TSP-treated chickens stored at 4°C for 7 days than for control chickens. In contrast, raw TSP-treated chickens in our study were not rated significantly higher than controls.

CONCLUSION

TREATMENT OF CHICKENS with 12% TSP for 15 sec or 0.5% lactic acid/0.05% sodium benzoate for 30 min did not adversely affect sensory quality of fried chicken. Furthermore, sensory quality of raw, whole chickens was not adversely affected by treatment with TSP. Use of TSP or LB as sanitizers for chickens

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Heat-Resistance of *Escherichia Coli* O157:H7 in Meat and Poultry as Affected by Product Composition

NAHED M. AHMED, DONALD E. CONNER, and DALE L. HUFFMAN

ABSTRACT

The effects of fat level and low fat formulation on survival of *Escherichia coli* O157:H7 isolate 204P heated in ground beef [7%, 10% and 20% fat], pork sausage [7%, 10%, and 30% fat], chicken (3% and 11% fat), and turkey (3% and 11% fat) were determined by D- and z-values. D-values for *E. coli* O157:H7 in lowest fat products were lower than in traditional beef and pork products ($P < 0.05$). Overall, higher fat levels in all products resulted in higher D-values. D_{60} values (min) ranged from 0.45–0.47 in beef, 0.37–0.55 in pork sausage, 0.38–0.55 in chicken and 0.55–0.58 in turkey. D_{55} and D_{50} values were respectively longer. Z-values ranged from 4.4–4.8°C. Product composition affected lethality of heat to *E. coli* O157:H7.

Key Words: meat, poultry, *E. coli*, fat content, thermal death

INTRODUCTION

ESCHERICHIA COLI occur among natural bacteria of the intestinal tract of humans and other animals (Drasar and Hill, 1974) but a limited number of types of *E. coli* are pathogenic. *E. coli* O157:H7 was established as a human pathogen in 1975 when it was isolated from a patient with perfuse diarrhea (Riley et al., 1983). Subsequently this pathogen was epidemiologically linked to consumption of contaminated food following outbreaks in Oregon and Michigan (Riley et al., 1983). Such outbreaks led to recognition of the enterohemorrhagic *E. coli* (EHEC) group of pathogenic *E. coli* (Padyhe and Doyle, 1991, 1992). Food is the primary route of transmission for *E. coli* O157:H7.

Cattle apparently have been a major reservoir of this pathogen. Undercooked ground beef and raw milk have been the foods most often associated with disease outbreaks (Padhye and Doyle, 1992). At least 16 documented foodborne disease outbreaks have been caused by *E. coli* O157:H7, of which, six have been attributed to ground beef (Mermelstein, 1993). One outbreak, from late 1992 to early 1993, received high news-media coverage and publicity. The cause of the outbreak was associated with undercooked hamburgers from a foodservice chain in several western states. Other outbreaks have been attributed to contaminated poultry (Ryan et al., 1986; Carter et al., 1987). *E. coli* O157:H7 readily colonized the ceca of chickens and was shed in feces for several months (Beery et al., 1985). *E. coli* O157:H7 was isolated from retail poultry in the U.S. (Doyle and Shoeni, 1987). However, the USDA indicated that of 6000 ready to cook poultry samples obtained directly from processing plants, none yielded the pathogen (Carosella, 1993). These findings indicate that poultry could be another reservoir for *E. coli* O157:H7 and that poultry products become contaminated during further processing, distribution and/or handling.

Undercooking or survival of the pathogen during cooking has been a contributing factor in most outbreaks (Mermelstein, 1993). Therefore, there is high interest in determining the heat resistance of *E. coli* O157:H7. This serotype is similar in many characteristics to other types of *E. coli* (Buchanan and Klawitter, 1992; Gibson and Roberts, 1986). However, it differs in its ability to ferment sorbitol and produce the β-glucuronidase enzyme, and exhibits considerable resistance to environmental stresses including acidic conditions, freezing and low growth temperatures (Conner and Hall, 1992, 1993, 1994; Conner and Kotrola, 1994). Thus, other characteristics, including heat resistance, may not be similar for all *E. coli* types.

Reportedly, *E. coli* O157:H7 has shown no unusual heat resistance, with D-values at 57–64°C ranging from 270–9.6 sec (Padhye and Doyle, 1992; Line et al., 1991; Shipp et al., 1992). However, intrinsic and extrinsic factors that affect heat resistance of *E. coli* O157:H7 have not been extensively studied. Isolates of *E. coli* O157:H7 vary considerably in heat resistance (Ahmed, 1994; Ahmed and Conner, 1992) and environmental conditions can increase D-values (Murano and Pierson, 1993). Moreover, few data have related the effects of meat product composition to survival of *E. coli* O157:H7 during cooking. Our objective was to determine the effects of species, fat content and low-fat formulation on heat resistance (survival) of *E. coli* O157:H7 in meat and poultry products.

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Table 1—Fresh pork sausage formulations

Ingredient (%)	Product 10% Fat	30% Fat	7% Fat[a]
Meat	94.60	94.60	76.90
Salt	1.630	1.630	2.030
Dextrose	0.250	0.250	0.313
Black pepper	0.160	0.160	0.200
Ground red pepper	0.030	0.030	0.038
Crushed red pepper	0.125	0.125	0.156
Carrageenan	0.000	0.000	0.350
Water	3.000	3.000	20.00

[a] AU Lean™ formulations.

Table 2—Proximate analysis of ground beef products

	Product 7% Fat[d]	10% Fat	20% Fat
Moisture (%)	72.6[a]	69.1[b]	61.9[c]
Fat (%)	7.2[c]	10.0[b]	19.1[a]
Protein (%)	19.1[b]	20.7[a]	18.2[c]

[a,b,c] Means within a row followed by different letters are different ($P < 0.05$).
[d] AU Lean™ formulation.

Table 3—Proximate analysis of pork sausage products

	Product 7% Fat	10% Fat	30% Fat
Moisture (%)	73.3[a]	70.9[a]	51.1[b]
Fat (%)	6.3[c]	11.3[b]	32.7[a]
Protein (%)	19.1[a]	16.8[b]	15.3[c]

[a,b,c] Means within a row followed by different letters are different ($P < 0.05$)
[d] AU Lean™ formulations

MATERIALS & METHODS

Inoculum preparation

E. coli O157:H7 strain 204P was obtained from M.P. Doyle, Univ. GA. and maintained on tryptic soy agar (TSA, Difco) at 4°C. Cell sus-

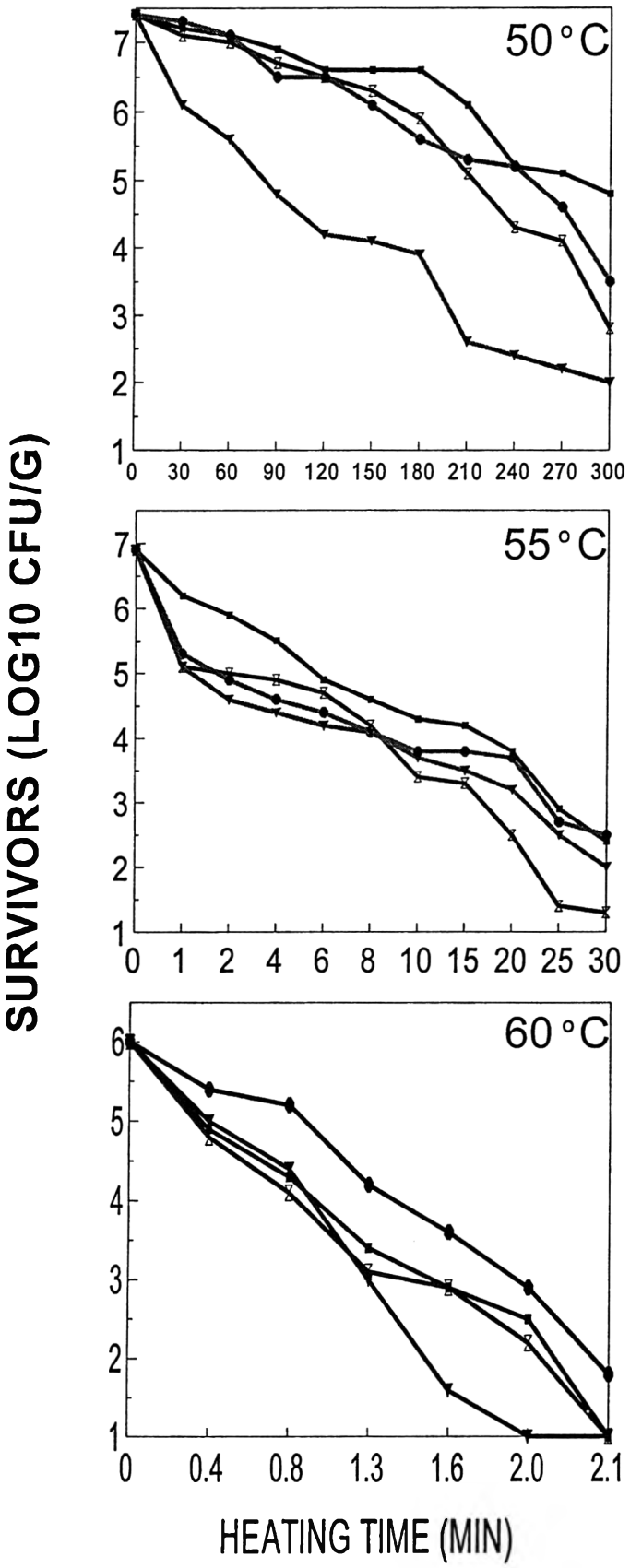

Fig. 1—Survivor curves of *Escherichia coli* O157:H7 strain 204P heated at 50°, 55° or 60°C in poultry meat. ▼ chicken 3% fat; ♦ chicken 11% fat; turkey 3% fat; ■ turkey 11% fat.

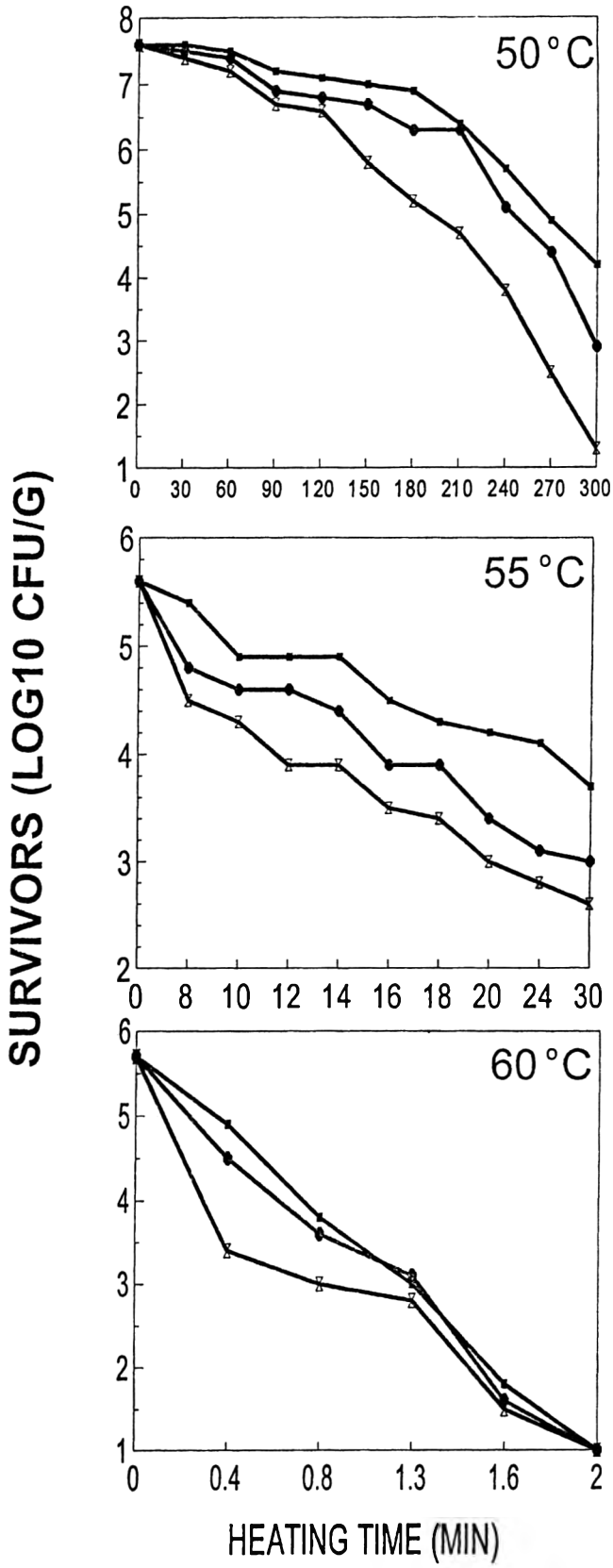

Fig. 2—Survivor curves of *Escherichia coli* O157:H7 strain 204P heated at 50°, 55° or 60°C in beef products. AU Lean™ beef 7% fat; ● Beef 10% fat; ■ Beef 20% fat.

pensions were prepared by two consecutive transfers in brain heart infusion (BHI, Difco) incubated for 24 hr at 37°C. Late stationary phase cells were harvested by refrigerated centrifugation at 14,000 × *g* for 10 min and washed twice in sterile Butterfield's phosphate buffer (pH 7.1). The inoculum suspension was enumerated by spiral plating (Spiral Biotech, Inc., Bethesda, MD) on TSA to verify the initial population for each experiment. Plates were incubated at 37°C for 24 hr before counting colonies.

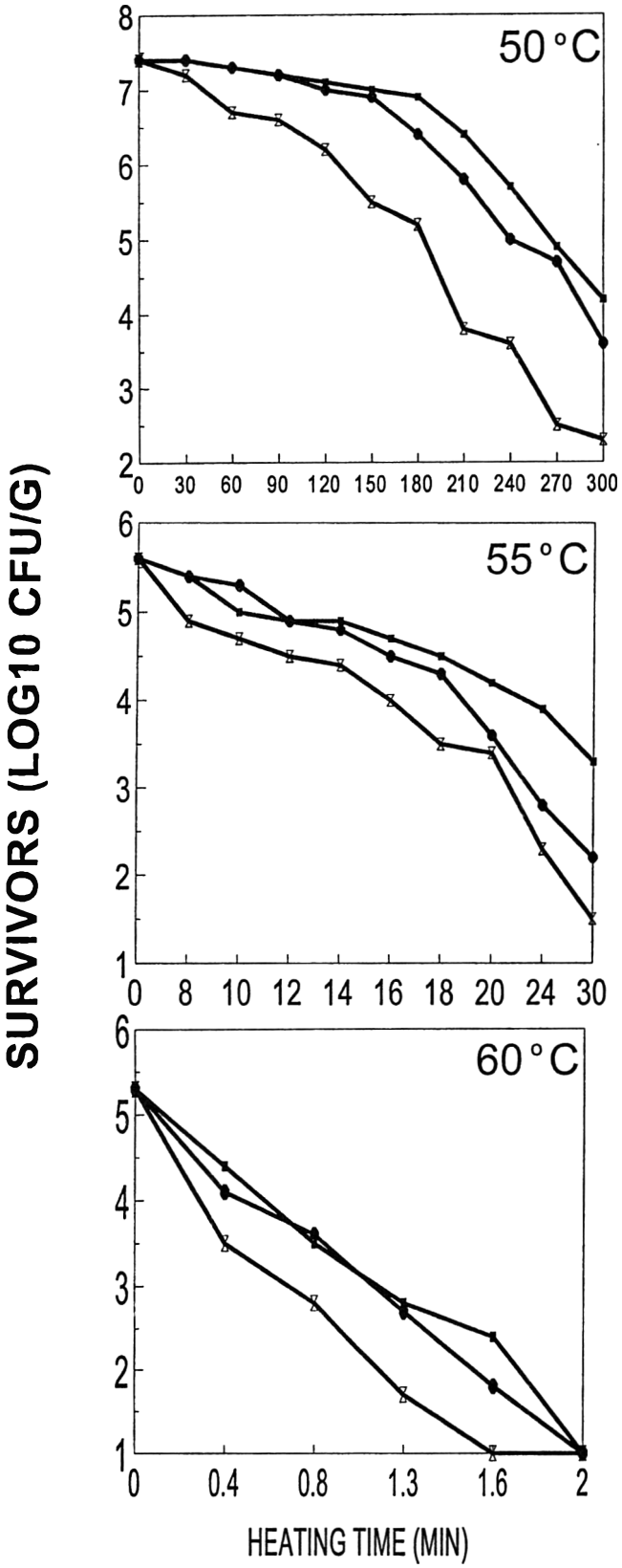

Fig. 3—Survivor curves of *Escherichia coli* O157:H7 strain 204P heated at 50°, 55° or 60°C in pork sausage products. AU Lean™ Pork sausage 7% fat; ● Pork sausage 10% fat; ■ Pork sausage 30% fat.

Table 4—D-value and regression values for *Escherichia coli* 0157:H7, isolate 204P, heated at 50°C in various meat products

Meat type	Fat content	Slope	Y-Intercept	Correlation coefficient	D-value (min)[d]
Chicken	3%	−0.015	6.450	0.88	65.24[b]
Chicken	11%	−0.009	7.716	0.87	105.5[a]
Turkey	3%	−0.014	7.339	0.89	70.41[b]
Turkey	11%	−0.009	7.365	0.88	115.0[a]
Beef[e]	7%	−0.018	7.324	0.95	55.34[c]
Beef	10%	−0.012	8.168	0.87	80.66[b]
Beef	20%	−0.011	7.577	0.97	92.67[a]
Pork sausage[e]	7%	−0.019	8.291	0.88	49.50[c]
Pork sausage	10%	−0.016	8.176	0.82	62.90[b]
Pork sausage	30%	−0.012	7.788	0.82	80.64[a]

[a-c] For each meat type (chicken, turkey, beef, or pork sausage), values with no common letter designation are different ($P < 0.05$).
[d] Calculated by linear regression. Mean value from three experiments in which at least four dwell times were used for linear regression analysis. Three replicates enumerated for each dwell time.
[e] AU Lean™ formulations

Table 5—D-value and regression values for *Escherichia coli* 0157:H7, isolate 204P, heated at 55°C in various meat products

Meat type	Fat content	Slope	Y-intercept	Correlation coefficient	D-value (min)[d]
Chicken	3%	−0.114	5.304	0.98	8.76[b]
Chicken	11%	−0.103	5.384	0.90	9.74[a]
Turkey	3%	−0.157	5.619	0.87	6.37[b]
Turkey	11%	−0.103	5.543	0.91	9.69[a]
Beef[e]	7%	−0.088	4.897	0.78	11.40[c]
Beef	10%	−0.066	4.868	0.87	15.30[b]
Beef	20%	−0.052	5.215	0.82	19.26[a]
Pork sausage	7%	−0.114	5.104	0.88	6.37[c]
Pork sausage	10%	−0.128	6.042	0.89	7.83[b]
Pork sausage	30%	−0.089	5.365	0.84	11.28[a]

[a-c] For each meat type (chicken, turkey, beef, or pork sausage), values with no common letter designation are different ($P < 0.05$).
[d] Calculated by linear regression. Mean value from three experiments in which at least four dwell times were used for linear regression analysis. Three replicates enumerated for each dwell time.
[e] AU Lean™ formulations

Table 6—D-value and regression values for *Escherichia coli* 0157:H7, isolate 204P, heated at 60°C in various meat products

Meat type	Fat content	Slope	Y-Intercept	Correlation coefficient	D-value (min)[c]
Chicken	3%	−2.616	6.132	0.78	0.38[b]
Chicken	11%	−1.834	5.334	0.76	0.55[a]
Turkey	3%	−1.809	5.619	0.89	0.55
Turkey	11%	−1.724	5.543	0.90	0.58
Beef[d]	7%	−2.182	5.128	0.85	0.45
Beef	10%	−2.206	5.533	0.95	0.46
Beef	20%	−2.125	5.536	0.96	0.47
Pork sausage[e]	7%	−2.669	5.042	0.90	0.37[c]
Pork sausage	10%	−2.134	5.330	0.93	0.46[b]
Pork sausage	30%	−1.809	5.213	0.86	0.55[a]

[a-b] For each meat type (chicken, turkey, beef, or pork sausage), values with no common letter designation are different ($P < 0.05$).
[c] Calculated by linear regression. Mean value from three experiments in which at least four dwell times were used for linear regression analysis. Three replicates enumerated for each dwell time.
[d] AU Lean™ formulations

Products

All meat products were packed in Whirl-Pak® polyethylene bags (Nasco Whirl-Pak), frozen and irradiated (10 KGy) to eliminate background microflora. Ground beef products tested included: (1) AU Lean™ (Huffman et al., 1991) containing 7% fat, 10% water, 0.5% carrageenan, 0.4% encapsulated salt and 0.2% hydrolyzed vegetable protein (HVP); (2) ground beef with 10% fat; and (3) ground beef with 20% fat. Pork sausage products (Table 1) included: (1) AU Lean™ with 7% fat; (2) sausage with 10% fat; and (3) sausage with 30% fat. Lean ground chicken and ground turkey containing 3% fat and "high" fat chicken and turkey containing 11% fat were also used.

Table 7—TDT curve data and resultant z-values for *Escherichia coli* 0157:H7, isolate 204P, heated in various meat products

Meat type	Fat content	Log_{10} D-value 50°C	55°C	60°C	z-value (°C)
Chicken	3%	1.81	0.94	−0.42	4.48
Chicken	11%	2.02	0.99	−0.26	4.38
Turkey	3%	1.85	0.80	−0.26	4.74
Turkey	11%	2.06	0.99	−0.24	4.35
Beef[a]	7%	1.74	1.06	−0.35	4.78
Beef	10%	1.91	1.18	−0.34	4.44
Beef	20%	1.97	1.28	−0.33	4.35
Pork sausage[a]	7%	1.69	0.80	−0.43	4.72
Pork sausage	10%	1.80	0.89	−0.34	4.67
Pork sausage	30%	1.91	1.05	−0.26	4.61

[a]AU Lean™ formulations

Proximate analysis

Proximate analyses were done on ground beef and pork sausage samples to determine fat, moisture, and protein content. Samples were ground twice using a mixer-grinder (Kitchenaid® model KSM90WH, St. Joseph, MD) equipped with a 4.5 mm grinding plate. Samples were stored frozen at −20°C, until analyzed for moisture, petroleum ether-extractable lipid, and protein (Kjeldahl) content using AOAC methods (AOAC, 1990).

Determination of heat resistance

Portions of each product were inoculated with *E. coli* O157:H7 cell suspension to provide ≈10^8 CFU/g, mixed, then aseptically placed (2g/tube) in thermal death time (TDT) tubes. Tubes were sealed, and completely immersed in a shaking water bath. Temperatures of tube contents were determined using a type K thermocouple (Science Electronics, Inc., Dayton, OH) connected to a data logger (Grant Squirrel Memory Logger, Science Electronics, Inc.). Timing of heat treatments began at the end of the come-up period (<1 min), with dwell times at each temperature:

50°C: 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300 min.
55°C: 0, 1, 2, 4, 6, 8, 10, 15, 20, 25, 30 min.
60°C: 0, 25, 50, 75, 100 sec, 2, 4, 6, 10 min.

Triplicate samples were removed after each dwell time and immediately cooled in an ice-water bath. Entire contents of each TDT tube were aseptically transferred to Butterfield's diluent, mixed, spiral plated (Spiral Biotech, Inc., Bethesda, MD, USA), on duplicate plates of phenol red agar (Difco) with 1% sorbitol (PRSA) (Ahmed and Conner, 1995). Samples not inoculated with *E. coli* O157:H7 were plated as controls.

D- and z-value calculations

Populations of survivors (log_{10} CFU/g) were plotted vs heating time for each temperature. D-values (min) were determined using linear regression analysis (SAS® Institute, 1988) for the best fit line of the survivor curve and following TDT procedures of the National Canners Association (1968). Z-values were calculated from thermal death time curves (log_{10} D-value vs temperature) as the absolute value of the reciprocal of the slope. At least five dwell times were used in calculation of D-values. Mean D-values were compared by analysis of variance using the general linear model procedure of SAS® (SAS® Institute, 1988). Means were separated using a least significance difference test (SAS® Institute, 1988). Significance was established at $P < 0.05$.

RESULTS & DISCUSSION

PROXIMATE ANALYSES indicated that the desired level of fat was present in the beef and pork products (Tables 2, 3), and that as fat content increased, moisture content decreased. Woolsey and Paul (1969) previously demonstrated this relationship between fat and moisture content. In the traditional beef and pork sausage products, as level of fat increased level of protein decreased ($P < 0.05$) (Tables 2, 3). Huffman and Egbert (1990) also reported an inverse relationship between fat content and protein. This trend did not hold true for the lowest fat beef and pork sausage products perhaps due to the additives used in formulations for those products.

Survivor curves were generated for each meat species at each heat treatment (Fig. 1, 2, 3). Greater numbers of *E. coli* O157:H7 were recovered from products with higher fat levels than from those with lower fat. Thus, resultant D-values were higher ($P < 0.05$) in products with higher fat contents. D-values and regression statistics for all products were compared (Tables 4 to 6). D_{50} values (Table 4) ranged from about 60 to 100 min for all products; D_{55} values (Table 5) ranged from around 6 to 10 min; and D_{60} values (Table 6) were all less than 1 min. Z-values for all products were similar, ranging from 4.35 to 4.78 (Table 7).

D-values we found were similar, but not identical to those reported by Line et al. (1991), Shipp et al. (1992), and Doyle and Schoeni (1984). Ahmed and Conner, (1992) demonstrated differences in heat resistance among different isolates of *E. coli* O157:H7. Based on their results, strain 204P, the most heat resistant isolate tested by Ahmed and Conner (1992), was utilized for our study. Thus, strain or isolate difference could account for differences in reported D-values. Methodology differences could also partially account for D-value differences. In our study PRSA was used as the recovery medium. PRSA has been shown to recover heat injured *E. coli* O157:H7 (Ahmed, 1994; Ahmed and Conner, 1995), whereas plate count agar with 1% pyruvic acid or MacConkey sorbitol agar failed to recover as many as 99% of sub-lethally-injured *E. coli* O157:H7. Thus, D-values derived with those media may not reflect true survival rates. Spread plate techniques (i.e. the Spiral Plate™ technique) can recover a higher number of cells than pour plate techniques (Swanson et al., 1992), which may also account for differences in D-values. Also, products we tested were free of background microflora, which could have influenced results.

Higher fat levels in all products resulted in higher D-values, consistent with other reports on this and other foodborne bacterial pathogens (Line, 1991; Doyle and Schoeni, 1987; Huang et al., 1992). Filppi (1973) showed that increasing the fat content of hamburger increased the D-value of poliovirus. Bacterial cells suspended in fat are typically more difficult to destroy than in aqueous medium, due to reduction in water activity. The water activity of the suspending medium in which spore forming or vegetative bacteria are heated influence heat resistance (Banwart, 1981; Murrell and Scott, 1966). Vegetative bacteria are more susceptible to heat in foods with higher water activities. Typically increasing fat content decreases water content, which alters heat transfer. This likely accounted for differences in survival of *E. coli* O157:H7 in the different products tested.

D-values for *E. coli* O157:H7 in the lowest fat products were lower than in traditional beef, pork, and poultry products ($P < 0.05$). These differences were pronounced in the pork sausage products, but were also clear in ground beef products. Such products had a higher moisture content (72.6%) and a lower fat content (7–8%) than the corresponding products. Because the lowest fat products contained higher moisture, the effect of heat on microbial cells was apparently greater. The reduced fat level and other related components (e.g. salt, increased moisture) of those products may have enhanced the lethality of heat on *E. coli* O157:H7.

Data indicate that cooking processes that provide an internal product temperature of 60°C for 2–3 min would provide a ≥5D kill of *E. coli* O157:H7 in all products tested. At lower temperatures (indicative of undercooking or partial processing), reduced fat products such as AU Lean™ ground beef and pork sausage could provide an additional margin of safety over traditional products.

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intended for frying, before presentation to the consumer appears to have good potential in the poultry processing industry.

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Storage Stability of Chicken as Affected by Map and Lactic Acid Treatment

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ABSTRACT

The effects of MAP and/or pretreatment with lactic acid buffer were studied on the shelf-life of broiler carcasses, under conditions simulating market storage (4 and 7°C) in the State of Kuwait. Pretreatment with lactic acid buffer increased treated carcass shelf-life by 6–7 days when stored at 4°C and 5–6 days at 7°C. Likewise, pretreatment of MAP carcasses with the same buffer extended the shelf-life to >36 and 35 days, compared with 22 and 13 days for the untreated MAP carcasses. Pretreatment of poultry with lactic acid buffer, with or without MAP, provides a potential alternative for improving the storage quality of poultry.

Key Words: poultry; microbes; storage stability; shelf life

INTRODUCTION

THERE IS RENEWED INTEREST in modified or controlled atmosphere storage of fresh meats. Developments in packaging materials and techniques have made such technology practical (Finn, 1982). The use of chemicals and techniques for reduction of the bacterial load on poultry meat and extension of shelf-life have been reported. Organic acids, such as lactic (Zeitoun and Debevere, 1990), citric, succinic, ascorbic and propionic acids (Izat et al., 1989), and/or their salts were evaluated in processing plants as decontaminating agents for reduction or elimination of spoilage and pathogenic organisms from poultry meat.

In addition to modified atmosphere-packaging (MAP), lactic acid has successfully extended the shelf-life of fresh meat and poultry (Mountney and O'Malley, 1965; Smulders, 1987; Van der Marel et al., 1988; Zeitoun and Debevere, 1990). The antimicrobial activity of lactic acid is a result of a decrease in pH and a specific antimicrobial effect of the undissociated molecule (Smulders, 1987). However, the inhibition of microbial growth also depends on the nature of the acidulant used and the dissociation constant (pK). The use of buffered lactic acid systems compared with unbuffered solutions also enhanced the decontaminating effect and increased the shelf-life of chicken legs (Van der Marel et al., 1988).

Our objective was to extend the shelf-life of chilled poultry through MAP and/or rinsing in buffered lactic acid. The effect of these treatments was compared with conventional poultry packaging.

MATERIALS & METHODS

TO STUDY THE SHELF-LIFE of MAP chicken pretreated with lactic acid buffer, more than 250 carcasses were obtained from a modern slaughterhouse and were divided into two groups. One group was packaged under modified atmosphere (70% CO_2, 5% O_2, and 25% N_2), and the second group was conventionally packaged, as practiced by the local poultry industry, in polyethylene bags perforated at the bottom for liquid discharge. Each group was subdivided into two subgroups. One was pretreated with lactic acid/sodium lactate buffer and one was not. The treated group was dipped for 1 min in a 10% (wt/vol) lactic acid/sodium lactate (0.902 mol/L lactic acid and 0.2082 mol/L sodium lactate, expressed as lactic acid in mol/L), as described by Zeitoun and Debevere (1990), and left to drain for 15 min before packaging. MAP carcasses were packed in heat-sealed multilayer gas barrier bags (Cryovac Co., Italy) permeability for CO_2 of 35 and for O_2 150 $cm^3/m^2/24$ hr, and a moisture vapor transmission rate of 20 $g/m^2/24$ hr, using a vacuum-packaging machine (Roescher Matrix, type VH-51/2, West Germany). All subgroups were stored at 4 and 7°C. Thus, 8 treatments were investigated: conventionally-packaged stored at 4°C (C-4), or at 7°C (C-7); pretreated with lactic acid before conventionally-packaged stored at 4°C (CL-4), or at 7°C (CL-7); modified atmosphere-packaged (MAP) with 70% CO_2 then stored at 4°C (4-70), or at 7°C (7-70); MAP pretreated with lactic acid then stored at 4°C (L4-70), or at 7°C (L7-70). Three carcasses representing each storage temperature were removed after 1, 3, 6, 8, 10, 13, 15, 17, 20, 22, 24, 27, 29, 31 and 34 days storage for microbiological and chemical analyses.

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Gas analysis

For analysis of CO_2 and N_2 levels in head space, a Hewlett-Packard gas chromatograph 5890 series II attached to a HP-CHEM station was used, equipped with a thermal conductivity detector. Helium (3.2 mL/min) was the carrier gas on a capillary column (Cp-Sil-8) 0.53 Id and 25m long. Inlet and detection temperatures were 80°C and column temperature was 27°C.

Microbiological analyses

The carcass-rinse method was used to obtain samples for microbiological analyses. Chicken carcasses were halved. One-half (≈500g) was placed in a sterile polyethylene bag (Seward Stomacher 400, UK) containing a 0.2% sterile lactose broth (1:1 wt/vol). The bags were shaken vigorously for 1 min, and aliquots transferred into sterile screw-cap dilution bottles. Decimal dilution series were set up for each aliquot and used for microbiological analyses. The other half was used for chemical analyses.

The homogenate was used for determination of psychrophiles on plate count agar (PCA-Oxcid) after 7 d incubation at 4°C; *Pseudomonas* on *Pseudomonas* base agar supplemented with selective agent X-108 after 2 days incubation at 22–25°C; *Lactobacillus* on Rogosa-agar (Difco) containing 1.32 ml/L glacial acetic acid (pH 5.4) for 2–3 days at 30°C in an anaerobic atmosphere (H_2 + CO_2); and Enterobacteriaceae on violet red glucose bile (VRGB) agar (Oxoid) after 24 hr incubation at 37°C.

Chemical analyses

Extract release volume (ERV) and free fatty acids (FFA) were determined according to the methods described by Egan et al. (1981) and Sawaya and Ruwaida (1989a, b).

Statistical analysis

For all determinations three replicates were used. The SAS means procedure which calculates values for both mean and standard deviation was used for data analysis (SAS Institute, Inc., 1985).

RESULTS & DISCUSSION

Spoilage indicators/metabolites

Changes in levels of ERV and FFA supported the results of sensory evaluation (Table 1). The increased levels of FFA and decreased value of ERV confirmed the relationship to spoilage of poultry meat (Sawaya and Abu Ruwaida, 1989a,b; Viehweg et al., 1989a,b). The antimicrobial effect of lactic acid may be the result of a decrease in pH and/or a specific antimicrobial

Table 1—Production of microbial spoilage indicators/metabolites in chicken carcasses during storage at different temperatures

Type of analysis	Storage time (days)	Temperature (°C) 4 Conv.[c]	4 Conv.L[d]	4 MAP[e]	4 L-MAP[f]	7 Conv.	7 Conv.L	7 MAP	7 L-MAP
ERV[a] (ml)	1	18.3	18.3	18.3	18.3	18.3	18.3	18.3	18.3
	3	14.0 ± 1.92	16.5 ± 0.71	18.0 ± 0.28	17.3 ± 0.35	14.5 ± 0.71	15.5 ± 0.28	16.0 ± 0.71	17.8 ± 0.42
	6	12.5 ± 2.12	15.5 ± 0.71	16.3 ± 1.77	17.0 ± 0.71	12.5 ± 0.00	13.8 ± 1.06	14.6 ± 1.84	16.5 ± 0.71
	8	12.0 ± 1.41	15.0 ± 1.41	15.5 ± 0.71	16.7 ± 0.71	—	12.8 ± 1.06	13.8 ± 0.21	16.0 ± 1.41
	10	—[g]	13.5 ± 2.12	14.8 ± 1.77	16.5 ± 0.42	—	11.8 ± 1.34	12.5 ± 1.41	15.5 ± 0.42
	13	—	12.5 ± 0.71	13.3 ± 0.35	16.0 ± 0.71	—	—	12.3 ± 0.21	15.0 ± 0.71
	15	—	12.5 ± 0.42	13.3 ± 0.35	15.5 ± 1.84	—	—	—	14.0 ± 2.83
	17	—	—	13.0 ± 2.83	15.0 ± 1.41	—	—	—	14.0 ± 0.00
	20	—	—	12.0 ± 0.35	15.0 ± 0.71	—	—	—	13.9 ± 0.92
	22	—	—	11.3 ± 0.35	14.4 ± 1.56	—	—	—	13.5 ± 1.41
	24	—	—	10.8 ± 0.71	13.8 ± 2.47	—	—	—	13.2 ± 1.13
	27	—	—	—	13.0 ± 0.71	—	—	—	12.8 ± 2.19
	29	—	—	—	12.8 ± 1.34	—	—	—	12.0 ± 0.71
	31	—	—	—	12.8 ± 0.35	—	—	—	12.0 ± 0.71
	34	—	—	—	12.8 ± 0.21	—	—	—	11.5 ± 0.00
	36	—	—	—	12.0 ± 0.71	—	—	—	10.8 ± 0.35
FFA (%)[b]	1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	3	0.4 ± 0.06	0.2 ± 0.04	0.3 ± 0.02	0.3 ± 0.03	0.2 ± 0.04	0.2 ± 0.03	0.4 ± 0.05	0.3 ± 0.03
	6	0.4 ± 0.02	0.3 ± 0.01	0.3 ± 0.04	0.4 ± 0.03	0.7 ± 0.07	0.3 ± 0.04	0.5 ± 0.04	0.5 ± 0.03
	8	0.7 ± 0.04	0.4 ± 0.03	0.4 ± 0.01	0.5 ± 0.01	—	0.8 ± 0.04	0.7 ± 0.02	0.6 ± 0.04
	10	—	0.8 ± 0.03	0.4 ± 0.01	0.5 ± 0.06	—	1.2 ± 0.05	0.8 ± 0.02	0.8 ± 0.01
	13	—	0.8 ± 0.04	0.5 ± 0.11	0.5 ± 0.02	—	—	1.1 ± 0.06	0.8 ± 0.06
	15	—	0.9 ± 0.07	0.6 ± 0.06	0.6 ± 0.09	—	—		1.0 ± 0.10
	17	—	—	0.7 ± 0.05	0.7 ± 0.04	—	—		1.1 ± 0.07
	20	—	—	0.7 ± 0.08	0.7 ± 0.01	—	—		1.1 ± 0.15
	22	—	—	0.9 ± 0.09	1.0 ± 0.06	—	—		1.2 ± 0.06
	24	—	—	0.9 ± 0.07	1.1 ± 0.03	—	—		1.4 ± 0.01
	27	—	—		1.1 ± 0.02	—	—		1.5 ± 0.04
	29	—	—		1.2 ± 0.12	—	—		1.8 ± 0.22
	31	—	—		1.2 ± 0.06	—	—		1.9 ± 0.04
	34	—	—		1.3 ± 0.04	—	—		2.0 ± 0.15
	36	—	—		1.9 ± 0.16	—	—		2.1 ± 0.08

[a] Extract release volume
[b] Free fatty acid
[c] Conventional air-packaged
[d] Conventional air-packaged with lactic acid/sodium lactate buffer
[e] Modified atmosphere-packaged
[f] Modified atmosphere with lactic acid/sodium lactate buffer
[g] Not determined due to spoilage

Table 2—Changes in pH of chicken carcasses during storage at different temperatures

Storage time (days)	Temperature (°C) 4 Conv.[a]	4 Conv.L[b]	4 MAP[c]	4 L-MAP[d]	7 Conv.	7 Conv.L	7 MAP	7 L-MAP
1	6.0	4.4	6.0	4.4	6.0	4.4	6.0	4.4
3	6.1	4.8	6.0	4.5	6.3	4.7	6.0	4.7
6	6.1	5.1	6.1	4.9	6.3	5.3	6.2	5.0
8	6.4	5.3	6.1	5.1	—	5.7	6.2	5.2
10	—[e]	5.6	6.1	5.3	—	5.9	6.3	5.3
13	—	6.1	6.2	5.3	—	—	6.3	5.4
15	—	6.1	6.2	5.4	—	—	—	5.4
17	—	—	6.2	5.5	—	—	—	5.5
20	—	—	6.3	5.6	—	—	—	5.7
22	—	—	6.3	5.7	—	—	—	5.7
24	—	—	6.4	5.7	—	—	—	5.8
27	—	—	—	5.9	—	—	—	5.9
29	—	—	—	5.9	—	—	—	6.1
31	—	—	—	6.0	—	—	—	6.2
34	—	—	—	6.1	—	—	—	6.2
36	—	—	—	6.1	—	—	—	6.2

[a] Conventional Air-Packaged
[b] Conventional Air-Packaged With Lactic Acid/Sodium Lactate Buffer
[c] Modified Atmosphere Packaged
[d] Modified Atmosphere With Lactic Acid/Sodium Lactate Buffer
[e] Not determined due to spoilage

effect of the undissociated molecule (Debevere, 1987; Smulders, 1987; Zeitoun and Debevere, 1990, 1992; Van der Marel et al., 1988).

Adding buffered lactic acid affected the carcass skin pH lowering it by > one unit. Although this decrease was recovered after a few days (Table 2), the pH remained lower for treated carcasses compared with untreated ones. Inclusion of 70% CO_2 in MAP carcasses did not affect pH. Thus, lowering the pH could not be a mechanism by which CO_2 inhibits growth of microorganisms, as was indicated by Brody (1989). However, the effect of CO_2 on the atmosphere of packaged carcasses may be due mainly to inhibition of specific spoilage microorganisms.

Change in levels of CO_2 in MAP carcasses

The CO_2 concentration in MAP-packaged carcasses decreased substantially after 3 days storage, then it stabilized and decreased slightly to the end of the experiment. This occurred in

Table 3—Percentage of CO_2 during storage of MAP-packaged chicken carcasses at different temperatures

Storage time (days)	MAP[a]		MAP/L[b]	
	4°C	7°C	4°C	7°C
1	72.3	72.3	72.3	72.3
3	38.3 ± 3.06	39.7 ± 5.69	43.0 ± 2.50	43.3 ± 2.08
6	35.0 ± 4.00	37.7 ± 1.53	36.0 ± 1.75	37.0 ± 1.73
8	31.0 ± 4.58	38.7 ± 3.06	36.0 ± 3.50	37.7 ± 0.58
10	32.0 ± 3.21	35.0 ± 1.50	30.5 ± 2.60	35.0 ± 0.75
13	30.7 ± 1.00	34.0 ± 4.51	34.0 ± 1.73	30.5 ± 1.05
16	ND	ND	ND	ND
18	28.7 ± 4.00	33.7 ± 3.21	29.0 ± 3.05	27.0 ± 2.25
20	28.3 ± 2.08	—	25.3 ± 2.00	25.7 ± 1.53
22	29.3 ± 0.50	—	26.0 ± 1.05	26.0 ± 2.00
24	30.7 ± 1.15	—	27.0 ± 1.00	27.0 ± 1.00
27	25.7 ± 3.51	—	32.0 ± 0.75	26.7 ± 2.31
29	—	—	25.3 ± 0.57	26.3 ± 2.89
31	—	—	25.0 ± 0.70	25.0 ± 1.72
34	—	—	22.0 ± 1.10	23.3 ± 2.08
36	—		21.3 ± 1.75	22.3 ± 1.15

[a] MAP = Modified Atmosphere-Packaged
[b] MAP/L = Modified Atmosphere-Packaged pretreated with Lactic Acid/Sodium Lactate Buffer
ND = Not Determined
— = Not determined due to spoilage

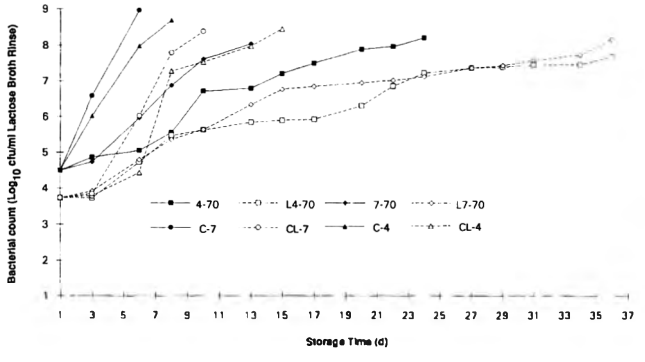

Fig. 1—Total counts of psychrotrophic bacteria in chicken carcasses stored at different temperatures: C-4 = conventionally-packaged stored at 4°C; C-7 = C stored at 7°C; CL-4 = C pretreated with lactic acid buffer stored at 4°C; CL-7 = CL stored at 7°C; MAP-4 = modified atmosphere-packaged stored at 4°C; MAP-7 = MAP stored at 7°C; MAP-L-4 = MAP pretreated with lactic acid buffer stored at 4°C; MAP-L-7 = MAP-L stored at 7°C.

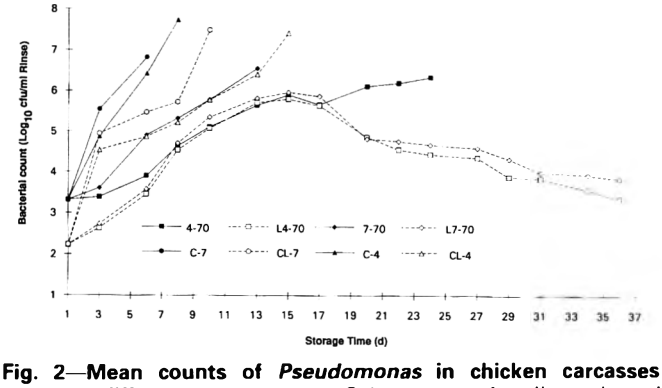

Fig. 2—Mean counts of *Pseudomonas* in chicken carcasses stored at different temperatures: C-4 = conventionally-packaged stored at 4°C; C-7 = C stored at 7°C; CL-4 = C pretreated with lactic acid buffer stored at 4°C; CL-7 = CL stored at 7°C; MAP-4 = modified atmosphere-packaged stored at 4°C; MAP-7 = MAP stored at 7°C; MAP-L-4 = MAP pretreated with lactic acid buffer stored at 4°C; MAP-L-7 = MAP-L stored at 7°C.

the presence or absence of lactic acid buffer (Table 3). The decrease of CO_2 concentration might be due mainly to its absorption into the meat and permeability through the bags. The

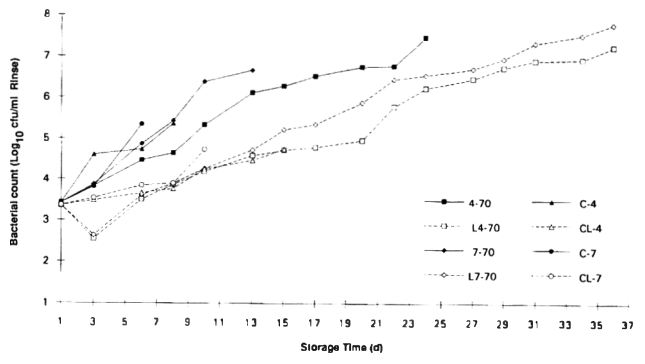

Fig. 3—Mean counts of *Lactobacillus* in chicken carcasses stored at different temperatures: C-4 = conventionally-packaged stored at 4°C; C-7 = C stored at 7°C; CL-4 = C pretreated with lactic acid buffer stored at 4°C; CL-7 = CL stored at 7°C; MAP-4 = modified atmosphere-packaged stored at 4°C; MAP-7 = MAP stored at 7°C; MAP-L-4 = MAP pretreated with lactic acid buffer stored at 4°C; MAP-L-7 = MAP-L stored at 7°C.

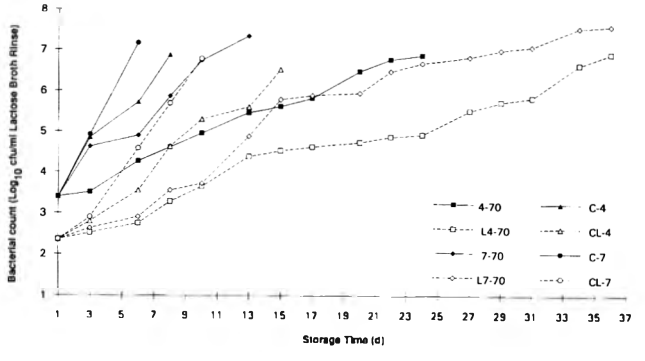

Fig. 4—Mean counts of *Enterobacteriacea* in chicken carcasses stored at different temperatures: C-4 = conventionally-packaged stored at 4°C; C-7 = C stored at 7°C; CL-4 = C pretreated with lactic acid buffer stored at 4°C; CL-7 = CL stored at 7°C; MAP-4 = modified atmosphere-packaged stored at 4°C; MAP-7 = MAP stored at 7°C; MAP-L-4 = MAP pretreated with lactic acid buffer stored at 4°C; MAP-L-7 = MAP-L stored at 7°C.

stability after 3 days might be due to the net result of less absorption (due to saturation) and/or evolution of CO_2 due to bacterial growth. O_2 and CO_2 are absorbed and metabolized by both the products and microorganisms, thus creating a partial vacuum inside the pack (Brody, 1989). The inclusion of N_2 gas in the head space would minimize the squeeze action from the meat, which causes excessive amount of drip or weep inside the package. Also, the inclusion of O_2 in MAP represents a safety factor for preventing growth of anaerobic pathogens (Brody, 1989).

Microbial growth. The combined effects of MAP CO_2:N_2:O_2 (70:25:5) and buffered lactic acid on growth of aerobic and facultative microorganisms was studied. Results were compared for Psychrotrophs, (Fig. 1) *Pseudomonas*, (Fig. 2) *Lactobacilli*, (Fig. 3) and Enterobacteriaceae (Fig. 4).

The mean bacterial count of psychrotrophs in the conventionally packaged carcasses increased from log 4.45 after 6 days at 4°C to log 7–8 after 5 days at 7°C. In MAP carcasses, about $\log_{10}$ 7–8 was reached after 22 days at 4°C and about 13 days at 7°C. When MAP carcasses were pretreated with lactic acid buffer, the mean count of about $\log_{10}$ 7–8 was reached after 35 days at 4 and after 36 days at 7°C. Using lactic acid buffer with conventionally-packaged carcasses almost doubled the shelf-life compared with that of the untreated conventionally packaged carcasses. The initial count was lowered by one log upon application of the buffer (Fig. 1). Zeitoun and Debevere (1990) reported that the use of 10% lactic acid buffer resulted in a reduction of psychrotrophic colony counts in poultry meat by

> one log compared with untreated meat. In our results, the initial count decreased by one log due to treatment with lactic acid. The rate of increase was much slower compared with that of the untreated meat, both for conventionally-packaged and MAP carcasses. Thus, the combined effect of the pretreatment of MAP (CO_2:N_2:O_2 70:25:5) carcasses with lactic acid buffer stored at 4°C substantially delayed the growth of psychrotrophs. They reached the maximum allowable level, (i.e., log 7), after 24 days compared with about 6 days for conventionally packaged carcasses stored at the same temperature. Although the mean counts of the psychrotrophic bacteria reached log 8 after 35 days, no off-odor or sliminess was observed in chicken carcasses, in agreement with Brody's observation (1981).

Pseudomonas is considered the main group of psychrotrophic bacteria responsible for poultry spoilage during chilling. Within 6–7 d, its count in the conventionally packaged carcasses stored at 4°C was > log 7 (Fig. 2). In MAP carcasses (CO_2:N_2:O_2 70:25:5), the *Pseudomonas* growth rate was suppressed, and the mean bacterial count reached log 6 only after 17 days. Pretreatment of MAP carcasses with lactic acid buffer gave similar results. Also the *Pseudomonas* bacterial count started to decrease after 15 d storage in the L4–70 and L7–70 chicken carcasses.

The *Lactobacilli* growth was slow in the conventionally packaged carcasses. Although the initial count was log 3.3, it did not exceed log 4 in C-4 and 5 in C-7 carcasses after 6 days storage. Inclusion of lactic acid buffer and/or MAP led to a slower *Lactobacilli* growth rate; the mean count reached log 5 only after 9, 14 and 20 days in 4–70, L7–70 and L4–70 carcasses, respectively. Thus, pretreatment of MAP (70:25:5) carcasses, with lactic acid buffer and storage at 4°C suppressed *Lactobacilli* growth for >20 days at 4°C. *Lactobacilli* count reached log 7 only after 35 days. However, evaluation of the sensory properties changed from very good to good after only 15 days storage and remained good up to 36 days when carcasses were stored at 4°C. Also, mean counts of *Lactobacilli* increased, whereas *Pseudomonas* started to decrease (Fig. 2, 3). This was due to the microaerophilic properties of the *Lactobacilli*, which can tolerate the modified atmosphere more than the *Pseudomonas*. This appeared to confirm the findings of Baker et al. (1985), that, at the initial stage, *Pseudomonas* comprised 90% of the total microorganisms in stored chicken. After 28 days, it comprised only 6% of the total organisms, whereas *Lactobacilli* increased. Moreover, organisms producing lactic acid did not result in spoilage, even though their numbers were high.

The Enterobacteriaceae growth was slower than that of the psychrotrophs and *Pseudomonas*. The initial count was about log 3.4/mL rinse, and reached log 5 after about 6 days in the case of the conventionally packaged carcasses stored at 4°C. However, it only reached log 5 after 10, 13 and 24 days in the 4–70, L7–70 and L4–70 carcasses, respectively (Fig. 4). Thus, pretreatment of MAP carcasses with lactic acid buffer and storage at 4°C resulted in retarding Enterobacteriaceae growth rate. After 35 days storage, the Enterobacteriaceae count was > log 6 and 7 only in L4–70, L7–70 carcasses, respectively. Other investigators (Gallo et al., 1988) reported that most psychrotrophic types of Enterobacteriacea in refrigerated fresh broilers were comprised of the species *Serratia* and *Proteus* but not *E. coli*.

CONCLUSIONS

PRETREATMENT OF FRESH CHICKEN carcasses with lactic acid buffer (10%) increased shelf-life to 13 when stored at 4 and 10 days at 7°C. This compared with 6–7 and 4–5 days for the untreated carcasses. Pretreatment of MAP carcasses with the same buffer could extend shelf-life to >36 days stored at 4°C and 35 days at 7°C compared with 22 and 13 days for untreated MAP carcasses (provided good manufacturing practices were followed). Extended shelf-life for both conventionally packaged or MAP carcasses could result in savings of energy costs needed for chilling. The shelf-life of MAP-L poultry superceded that of vacuum-packaged carcasses.

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Volatile Compounds in Processed Duck Fillet

S. LESIMPLE, L. TORRES, S. MITJAVILA, Y. FERNANDEZ, and L. DURAND

ABSTRACT

Volatile components of smoked and dry-cured, smoked, brined or raw duck fillets were fractionated by simultaneous distillation-extraction before GC/MS. Volatile compounds (62) identified were phenols, alcohols, ethers, aldehydes, ketones, hydrocarbons, acids, esters, terpenes and N- and S-compounds. Some compounds (34) were related to the smoke process. Most of these flavor compounds had been identified in smoked meat products and 7 are reported for the first time. The formation of aldehydes and alcohols mainly results from oxidative decomposition of membrane polyunsaturated fatty acids.

Key Words: duck fillet, smoking, flavor compounds, hydrocarbons, aldehydes.

INTRODUCTION

MEAT FOOD PRODUCTS were historically preserved by exposure to the aromatic smoke of burning hardwoods. Smoked food products are still desired for their typical aromatic flavors. Ducks grown for the specific production of "foie gras" (duck liver) are well known for the excellent quality of their meat. In particular, the fillet covered by the subcutaneous fatty panicle, (called "magret" in French cuisine) can be improved in flavor quality by smoke-curing.

The flavor of this processed fillet is related to exogenous and endogenous compounds and their distribution between the fatty panicle and the muscle. Endogenous compounds result from a transformation of lipids, especially phospholipids of muscle tissue membranes, occurring during curing and drying of meat (Ladikos and Lougovois, 1990; Ajuyah et al., 1993). During curing, these fatty acids are subjected to a peroxidation process catalyzed by hemoglobin, myoglobin and cytochrome to yield alkenals, dienals, alcohols and ketones, generally considered to contribute to the aroma of processed meats (Banks, 1952). Exogenous compounds arise from the smoking process and use of appropriate condiments.

Although more than 300 compounds of exogenic origin have been identified in smoke, only a very small number have been recovered in smoked food products (Hamm, 1977; Ohshima et al., 1989) to which they contribute desired sensory properties (Hassan, 1988) such as coloration (Ruiter, 1979), texture and flavor (Sink, 1979; Clifford et al., 1980; Shiau and Chai, 1985). Such compounds are principally phenols, aldehydes, ketones, some acids and hydrocarbons. Factors inherent in the smoking process can influence the nature and ratio of such compounds in food products. Such factors include: kind of wood (Lantz and Vaisey, 1970), type of exposure to smoke (direct or indirect), temperature (Deng et al., 1974; Clifford et al., 1980), duration of smoking, hygrometry (Chan, 1975), air flow rate in the smoking chamber and presence of oxygen (Girard, 1965; Sirami, 1981). Intrinsic factors which influence the nature and ratio of volatile compounds include water, protein and fat contents of the meat. They are susceptible to affect interactions with smoke constituents (Daun, 1979; Clifford et al., 1980), and hence, sensory qualities of final products.

Our objective was to isolate volatile compounds that are present in processed duck fillets by simultaneous distillation extraction and to analyze such compounds by GC/MS. We also considered possible origins of such compounds with respect to different technological treatments.

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MATERIALS & METHODS

Treatment of duck fillets

After cutting carcasses, raw duck fillets (with subcutaneous fatty panicle) were subjected to three treatments: brining, smoking and drying. Brining consisted of a dry rub of raw fillets with salt (20g/kg) and pepper (2g/kg) followed by curing at 4°C for 12 hr. After curing, the duck fillet was smoked in an indirect way according to a classic procedure using a device where the smoke generator and the smoking chamber were separated. The oven which produced the smoke consisted of a circular metallic plate on which beech wood shavings were regularly dropped and pyrolyzed, at temperatures ≤300°C. The smoke flux was directed toward the smoking chamber through a diverting pipe where solid tar particles and less volatile compounds were retained. The smoke entered the smoking chamber via three openings. The duck fillets, hung in the chamber, were smoked at 20°C for 3 hr. After smoking duck fillets were placed in a thermostated chamber kept at 12°C where the air was constantly stirred while relative humidity was maintained at 80 to 85%. After 10 days, the duck fillets were packed under vacuum.

Batches of 6 duck fillets (total weight ~1 kg) were collected at each step of preparation procedure. Each batch of meat was cut and crushed in a grinder (mod. R-6, Robot-Coupe s.a. 94170 Le Perreux, France). From the resulting sample a fraction (100 g) was weighed and used immediately for extraction.

Distillation/extraction procedure

Distillation and extraction were carried out simultaneously in a Likens-Nickerson apparatus (Nickerson and Likens, 1966). In a first flask, 100g crushed meat were mixed with 300 mL distilled water. A second flask was filled with 80 mL diethylether. Such solvent provided an acceptable compromise for extraction of polar and nonpolar compounds (Sakakibara et al., 1990; Tanchotikul and Hsieh, 1991). The two solutions were heated under reflux. Vapors were condensed in a reflux condenser in which the circulating fluid was a water/ethylene glycol mixture allowing cooling temperatures of 0 to 5°C. The volatile products which formed continuously were extracted by vapors of the extractor solvent and concentrated in a reduced volume of solvent.

After dehydration with anhydrous Na_2SO_4, the solvent was reduced in volume to a few mL under reduced pressure. Then extracts were dried under a gentle stream of nitrogen and weighed. The weight of extracts generally ranged between 10 and 15 mg. These residues were recovered with 0.3 mL diethylether and analyzed by gas chromatography coupled with a mass spectrometer (GC/MS).

Experimental device and procedure

Analyses were carried out on a Hewlett-Packard 5890A gas chromatograph connected to a Hewlett-Packard 5971 mass selective detector. Extract (1 µL) was injected in splitless mode into a fused silica capillary column (BP5, 25 m length, 0.25 mm i.d., 0.25 µm film thickness, cross-linked 5% diphenyl dimethyl siloxane, SGE France). Analysis of extracts was carried out with a temperature program of (1) constant 50°C for 4 min; (2) increase from 50° to 290°C at 7°C/min; (3) constant 290°C for 15 min.

Identifications were carried out by comparison of mass spectra of compounds with those stored in the NBS mass spectral library. The retention

times of compounds were compared with authentic standards analyzed under comparable conditions. Most compounds identified originated from smoking and curing but a few of them may be artifacts arising during chopping and distillation.

RESULTS & DISCUSSION

Evaluation of volatiles

A semi-quantitative time course (2, 5, 10 and 15 hr) performance of steam distillation-extraction was investigated by GC/MS for different classes of organic compounds on the same sample of smoked and dried duck fillet (Table 1). Each column represented the cumulative chromatographic signal corresponding to the given time. With reference to the signal obtained after 15 hr, exogenous compounds such as phenols, ethers, terpenes were extracted at 85% after 5 hr. For the other classes of compounds, especially aldehydes and acids resulting from a transformation of lipids, the total amount recovered increased regularly with time. Such compounds could have possibly been partly created during the distillation-extraction process. These preliminary results enabled us to establish 5 hr as the time required to achieve reasonable recovery of exogenous compounds, limiting the presence of newly formed compounds which may have originated from endogenous lipids.

Extracts composition

A total of 62 compounds were identified from the extracts. These compounds included the following: phenols, alcohols, ethers, aldehydes, ketones, hydrocarbons, terpenes, sulfur containing compounds, fatty acids and esters (Table 2).

The chromatograms from smoked dry-cured (Fig. 1) and smoked duck fillets exhibited many more peaks than those from raw and brined duck fillets. The presence of 10 phenolic compounds was directly ascribed to smoking. The combustion of cellulose, hemicellulose and lignin of the wood produces almost all phenolic compounds. Phenols such as 0- and m-cresol, guaiacol, 4-methylguaiacol, 4-ethylguaiacol, xylenols came from smoke and were only found in products smoked at low temperature, due to their relatively high vapor pressures. Other phenolics undetected in our study, such as syringaldehyde and acetovanillone, have been more commonly encountered in products smoked at high temperatures, due to their high boiling points (Potthast, 1977, 1978). Syringol, found in our study, was much more abundant than other phenols mentioned. This was due to the nature of the wood used in our smoking procedure (beech). Hardwoods are characterized by the presence of methoxy or dimethoxy groups on aromatic compounds found in lignin. Whereas guaiacols prevail in the combustion of softer woods, hard woods produce a mixture of guaiacols and syringol (Gilbert and Knowles, 1975). Phenols are considered as potential aromatics (Bratzler et al., 1969). In spite of its high boiling point, eugenol was present in all smoked meats and its contribution to the aroma seemed higher than that of guaiacol (Potthast, 1978). Guaiacol was characteristic in the smoked taste, whereas the smoked smell has been attributed to syringol (Daun, 1979). Earlier studies have shown that mixtures of these two compounds existing in proportions corresponding to those found in condensed smoke residues were only slightly reminiscent of the smell and taste of smoked products (Gilbert and Knowles, 1975). The sensory properties of the final smoked products were due to a complex mixture of compounds coming from the smoke combined with the initial flavor of the food product. Reaction products between smoke constituents and surface proteins of the food are also responsible for some of the sensory properties (Gilbert and Knowles, 1975; Daun, 1979).

Table 1—Cumulative chromatographic area time course for compounds extracted from processed duck fillet

Chemical compounds	2 hr	5 hr	10 hr	15 hr
Phenols (10^6)	13983	20710	22600	23901
Alcohols (10^6)	588	1150	1415	1661
Ethers (10^6)	164	294	319	333
Aldehydes (10^6)	17370	26485	34172	41679
Ketones (10^6)	2640	4381	5418	6161
Hydrocarbons (10^6)	1683	3011	3413	3613
Acids (10^6)	13107	38645	47837	56669
Terpenes (10^6)	2600	4714	5167	5367
N- and S-compounds (10^6)	510	642	683	719

Phenols and aldehydes exert an antiseptic effect enhanced by desiccation of the surface during smoking and drying (Hess, 1929; Gibbons et al., 1954; Sofos and Maga, 1988; Ismaiel and Pierson, 1990). Phenolic compounds with low boiling points are known to inhibit bacterial growth by prolongation of the latent phase. In addition to contributions to sensory properties, phenolic compounds such as syringol and eugenol, present in smoked fillets, also inhibit oxidative degradation of fatty products (Barylko-Pikielna, 1977). Phenols are adsorbed onto smoked products by formation of hydrogen bonds between their hydroxyl groups and constituents of collagen (Daun, 1979).

Except for vanillin, produced through smoking, and piperonal, from pepper, all remaining aldehyde-type products were detected in the 4 extracts investigated (Table 1). Among those identified, (Z)-9-hexadecenal, hexadecanal, and (Z)-9-octadecenal, were derived from oxidative decomposition of fatty acids within the product. On the other hand, (E, E)- 2,4-decadienal, and 2,6-nonadienal resulted from the respective oxidation of 6-ω and 3-ω polyunsaturated fatty acids (Finley and Given, 1986; Koelsch et al., 1991; Ajuyah et al., 1993).

Among the three nonaromatic alcohols, tetradecanol and hexadecanol were present in brined, smoked, and smoked dry-cured duck fillets. They resulted from enzymatic reduction of aldehydes formed during oxidative degradation of fatty acids (Tanchotikul and Hsieh, 1989; Barbieri et al., 1992; Ajuyah et

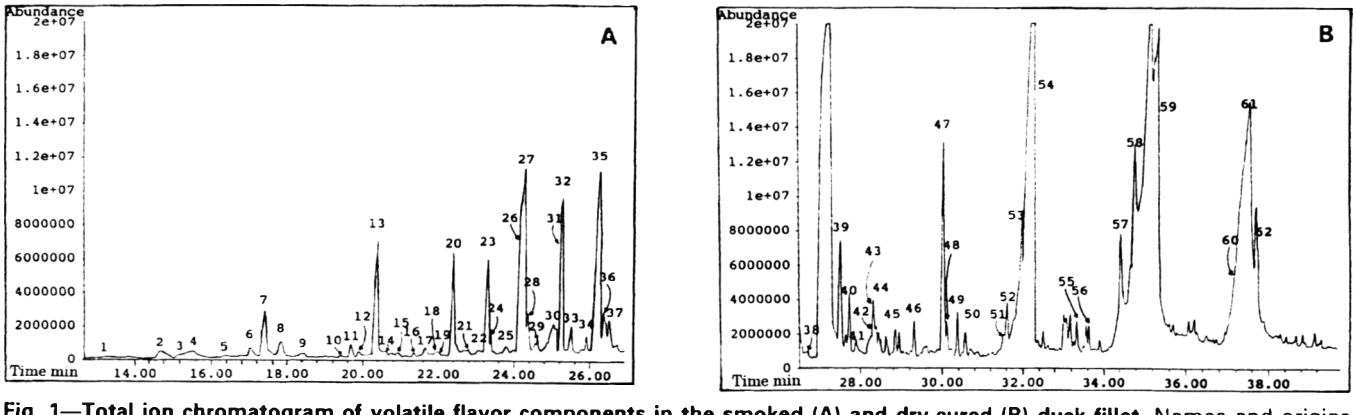

Fig. 1—Total ion chromatogram of volatile flavor components in the smoked (A) and dry-cured (B) duck fillet. Names and origins are given in Table 2.

Table 2—Volatile components of duck fillets[a]

Peak no.	Compound	ID[b]	Smoked dried-cured duck fillet	Smoked duck fillet	Brined duck fillet	Raw duck fillet
	Phenols					
2	phenol	RT, GC/MS	+	+	–	–
6	o-cresol	RT, GC/MS	+	+	–	–
7	guaiacol	RT, GC/MS	++	++	–	–
8	m-cresol	RT, GC/MS	+	+	–	–
10	3-ethylphenol	GC/MS	+	+	–	–
11	2,4-dimethylphenol	RT, GC/MS	+	+	–	–
13	4-methylguaiacol	RT, GC/MS	++	++	–	–
14	2,3-dimethylphenol	RT, GC/MS	+	+	–	–
18	2-ethyl-6-methylphenol	GC/MS	+	+	–	–
20	4-ethylguaiacol	RT, GC/MS	++	++	–	–
26	eugenol	RT, GC/MS	+	+	–	–
27	syringol	RT, GC/MS	++	++	–	–
28	2-methoxy-4-propylphenol	GC/MS	+	+	–	–
31	isoeugenol	RT, GC/MS	+	+	–	–
45	allylsyringol	RT, GC/MS	+	+	–	–
	Alcohols					
3	2-ethyl-l-hexanol	GC/MS	+	+	+	+
5	1,3,5-benzenetriol	GC/MS	+	+	–	–
16	2,3,5-trimethyl-1,4-benzenediol	GC/MS	+	+	–	–
46	(−)-spathulenol	GC/MS	+	+	–	–
47	tetradecanol	RT, GC/MS	++	+	+	–
52	hexadecanol	RT, GC/MS	+	+	+	+
	Ethers					
12	1,4-dimethoxybenzene	GC/MS	+	+	–	–
29	1,2,3-trimethoxy-5-methylbenzene	GC/MS	+	+	–	–
	Aldehydes					
21	2,6-nonadienal	RT, GC/MS	+	+	+	+
22	(E,E)-2,4-decadienal	RT, GC/MS	+	+	+	+
25	piperonal	GC/MS	+	+	+	–
33	vanillin	RT, GC/MS	++	++	–	–
49	(Z)-9-hexadecenal	RT, GC/MS	+	+	+	+
54	hexadecanal	RT, GC/MS	+++	+++	+++	+++
57	(Z)-9-octadecenal	RT, GC/MS	++	++	++	++
	Ketones					
4	2-hydroxy-3-methyl- 2-cyclopenten-1-one	GC/MS	+	+	–	–
9	2,2-dimethyl-1,3-cyclopentanedione	GC/MS	+	+	–	–
23	1-(2-hydroxy-5-methylphenyl)-ethanone	GC/MS	++	++	–	–
39	1-(2,6-dihydroxy-4-methoxyphenyl)-ethanone	GC/MS	++	++	–	–
40	1-(4-hydroxy-3-methoxyphenyl)-2-propanone	GC/MS	+	+	–	–
55	2-dodecanone	GC/MS	+	+	+	–
	Hydrocarbons					
1	1,2,3-trimethylbenzene	GC/MS	+	+	+	–
24	δ-elemene	GC/MS	+	+	–	–
36	tetradecane	RT, GC/MS	+	+	+	+
37	pentadecane	RT, GC/MS	+	+	+	–
38	2-methyl-9H-fluorene	GC/MS	+	+	–	–
42	hexadecene	RT, GC/MS	+	+	+	–
43	hexadecane	RT, GC/MS	+	+	+	–
48	octadecane	RT, GC/MS	+	+	+	–
50	1,2,3-trimethyl-(E)-4-propenylnaphthalene	GC/MS	+	+	–	–
	Acids					
30	decanoic acid	GC/MS	+	+	–	–
35	vanillic acid	RT, GC/MS	++	++	–	–
44	dodecanoic acid	RT, GC/MS	+	+	+	+
53	tetradecanoic acid	RT, GC/MS	+	+	+	+
58	9-hexadecenoic acid	RT, GC/MS	++	++	++	++
59	hexadecanoic acid	RT, GC/MS	+++	+++	+++	+++
60	(Z)-9,12-octadecadienoic acid	RT, GC/MS	++	++	++	++
61	(Z)-9-octadecenoic acid	RT, GC/MS	+++	+++	+++	+++
62	octadecanoic acid	RT, GC/MS	+	+	+	+
	Ester					
56	hexadecanoic acid, methyl ester	RT, GC/MS	+	+	+	+
	Terpene					
32	caryophyllene	RT, GC/MS	++	++	+	–
34	α-caryophyllene	RT, GC/MS	+	+	+	–
	N- and S-compounds					
15	benzothiazole	GC/MS	+	+	–	–
17	1-methoxy-4-(methylthio)-benzene	GC/MS	+	+	–	–
19	1-(ethylthio)-3-methylbenzene	GC/MS	+	+	–	–
41	2,5-dibutylthiophene	GC/MS	+	+	–	–
51	2,4-diphenyl-1H-pyrrole	GC/MS	+	+	–	–

[a] The signs (+) or (−) indicate the presence or the absence of a given compound in the chromatogram for each of the four extracts. (++) indicates an important relative abundance of the compound; (+++) indicates an important abundance of the compound.

[b] Indicates the identification procedure: RT (retention time) and/or MS (mass spectrometry).

al., 1993). 1, 3, 5-Benzenetriol and 2, 3, 5-trimethyl- 1, 4-benzenediol were formed during the smoking process.

Among ketones, only 2-dodecanone was found in brined duck fillet, whereas the other three products detected in smoked and smoked dry-cured duck fillets resulted from the smoking. 2,2-Dimethyl-cyclopentanedione has been previously reported in liquid condensed smoke residues and in smoked bacon (Wittkowski et al., 1990). Aldehydes and ketones contribute to the flavor of smoked products and also to their color. The brown coloration of smoked products is due to reactions involving carbonyl groups of smoke components and amine functions of proteins. This tends to reduce the nutritional protein quality of such products due to partial loss of amino acids like lysine and tryptophane (Munro and Morrison, 1965; Bhuiyan et al., 1986). Studies have indicated losses of lysine reached 44% in smoked beef (Daun, 1979; Steiner-Asiedu et al., 1991).

The two ethers were 1,4-dimethoxybenzene, and 1,2,3-trimethoxy-5-methylbenzene. They were present in the extracts of smoked and smoked dry-cured duck fillets. They have been reported in dried smoked fish (Sakakibara et al., 1990). Noticeably, most aliphatic hydrocarbons were absent from raw duck fillets. Their presence in further stages of preparation may be related to curing and transformation during smoking and drying (Ladikos and Lougovois, 1990). Among the polycyclic aromatic hydrocarbons detected, traces of 2-methyl-9H-fluorene and 1, 2, 3-trimethyl-(E)-4-propenylnaphthalene, which have been commonly reported in smoked products (Hamm, 1977; Grimmer and Jacob, 1987; Larsson et al., 1988) were noted.

Sesquiterpenoids, caryophyllene and its isomer, detected in the three extracts, came from pepper added during brining (Narasimhan et al., 1992; Richard and Jennings, 1971). Fatty acids identified in this work included 9-hexadecenoic acid, tetradecanoic acid, hexadecanoic acid, 9-octadecenoic acid, 9,12-octadecadienoic acid and octadecanoic acid. The presence of fatty acid esters in brined, smoked and smoked dry-cured duck fillets is related to the drying and curing processes (Barbieri et al., 1992).

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Time/Temperature Profiles of Smoked Salmon Packaged with Cooling Gel and Shipped at Ambient Temperature

CESARETTIN ALASALVAR and PAUL NESVADBA

ABSTRACT

The latent heat capacities of three different commercial cooling gels and ice were measured by Differential Scanning Calorimetry (DSC). Their cooling ability and relative merits were compared. DSC was also used to measure the thawing onset temperatures and the latent heat capacities of salmon. The latent heats of the cooling gels were 323.4 ± 0.2 J/g, 323.4 ± 0.4 J/g, and 323.6 ± 0.1 J/g, whereas that of ice was 334.5 ± 0.04 J/g. The time-temperature profiles of fish were followed to determine how long the temperature of the products could be maintained <8°C. We could predict by computer modeling the time-temperature profile of smoked chilled salmon while in shipment.

Key Words: computer modeling, temperature prediction, thermophysical properties, cooling gels

INTRODUCTION

SMOKED SALMON is packaged and shipped at ambient temperature throughout the UK. The market for smoked salmon increased sharply following growth of production of farmed salmon (48689 MT in 1993; Fish Farmer, 1994). The product has changed from a costly luxury item to one which is readily available in supermarkets. The market has become more competitive resulting in processors examining better ways of preparing and distributing smoked salmon. Chilling during mail shipment is an area requiring improvements.

The US FDA (1992) acknowledged that "Ice-berg" (a water-soluble synthetic co-polymer with cold retention ability) was nontoxic and nonhazardous and could be used as a coolant for perishable products during distribution. Some companies (Shetland Smokehouse and Ace Dry Ice & Gel Packs, UK) indicate that "Ice-berg" has a cold retention higher than any starch-based gel or water. They claimed "Ice-berg" cold pack rating was 409.5 J/g, which seemed unlikely and was therefore investigated.

The perishability of fish makes temperature control very important. The temperature of packaged foods shipped at ambient temperatures should not exceed 8°C for safety during transit (UK, 1990; Dept. of Health, 1990). Temperatures >8°C may allow microbial growth and lead to health problems for consumers, although the Public Health Laboratory Service and the Department of the Health have no records of food poisoning in the UK related to such products at ambient temperatures (MAFF, 1991). With lack of apparent time-temperature control during distribution and the trend towards producing lightly processed foods (less salt, smoke and drying), there was a need to investigate such ambient shipped products.

Our objectives were to measure the latent heat of fusion of "Ice berg" and other gels, compare the cooling ability and relative merits of coolants at ambient temperature, and model the profiles of frozen and chilled foods during shipment.

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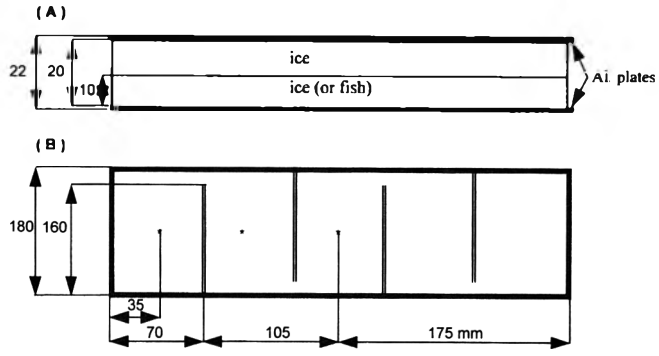

Fig. 1—A schematic of (A) ice or gel block and (B) vertical sealed polyethylene bag.

MATERIALS & METHODS

ICED ATLANTIC SALMON (*Salmo salar*) were delivered from Aberdeen Fish Dock to the Food Science Laboratory (FSL)-Torry. They were smoked using the Torry Kiln, vacuum packed and stored in a chilled room at 4–5°C until required for experimental work. "Ice-berg" (A), "sorbagel" (B) and "ice-keeper" (C) were supplied by Shetland Smokehouse (Shetland), J.P.E. International Corp. (Brooklyn N.Y., 11211) and Shetland Smoked Salmon (Shetland), respectively.

The gels were prepared by mixing 4.158g of dry cooling gel (in powder form) with 630 mL of water to fill a polyethylene bag (350 mm × 180 mm × 10 mm). Two such bags were used in each experiment with three thermocouples and three thermistors sandwiched between them. This assembly was placed between two aluminum plates in order to achieve more uniform temperature on the surfaces. In bags with water, in order to prevent ice movement during melting, internal partitions were made by welding the walls of the bags (Fig. 1).

Mail order firms typically use a polystyrene box (390 mm × 230 mm × 68 mm) and this was used in our experiments. Both sides of the box were covered with aluminum plates so that heat could be distributed evenly. In order to measure the correct surface temperature, thermistors were coated with heat sink compound at the point of contact with plates. The polystyrene box was placed inside a tightly fitting outer cardboard box (2 mm thickness) to prevent damage in the mail.

Latent heat and freezing point determination by differential scanning calorimetry

Perkin Elmer DSC Datalab 7 was used with samples of 5–10 mg, encapsulated in aluminum containers. The scanning rate was 10°C/min in the range −35 to 10°C. Indium was used for calibration of energy and temperature scales.

Time-temperature measurement of the gels and ice

After blast freezing to −35°C, the gel packs were placed inside the polystyrene box and then stored at ambient temperature (25 ± 1°C) during thawing. The data from thermocouples and thermistors were recorded at 15 min intervals by a SP25 Dataprinter (with a printer output Digitron) and "Tinytalk" Datalogger (Orion components, Ltd. Chichester, UK), respectively.

Time-temperature measurement of fish during mail shipment

The gel A, blast frozen to −35°C, was placed on the top of minced smoked salmon pack (≈700 g) with the same major dimensions. The

Table 1—Latent heat capacities and thawing onset temperatures of the coolants and salmon

Samples	Latent heat (J/g)	Thawing onset (°C)
Indium	28.45 ± 0.0	156.60 ± 0.01
Water (Ice)	334.50 ± 0.04	−0.01 ± 0.05
Coolant A	323.41 ± 0.2	−0.71 ± 0.1
Coolant B	323.37 ± 0.4	0.1 ± 0.06
Coolant C	323.63 ± 0.1	−0.66 ± 0.05
Fresh salmon	210.38 ± 0.05	−2.56 ± 0.06
Smoked salmon	119.45 ± 0.12	−11.28 ± 0.15
Minced smoked salmon	118.70 ± 0.16	−10.12 ± 0.17

Means of triplicate determinations

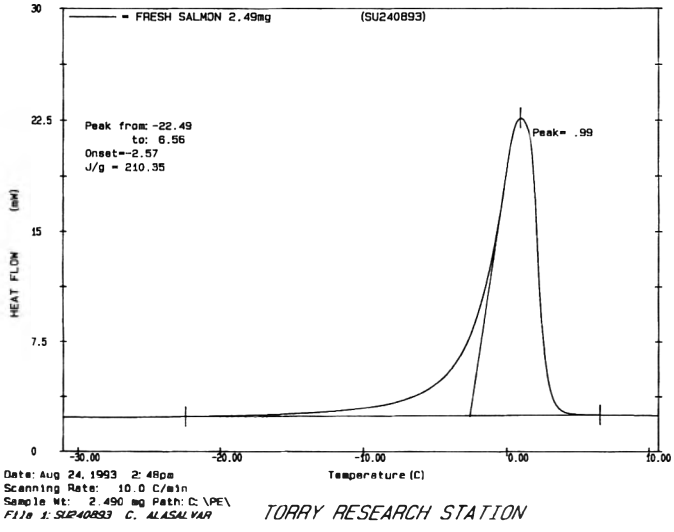

Fig. 2—Latent heat and thawing onset temperature of fresh salmon.

assembly was stored at 4–5°C before packing. Thermistors were attached between the gel A and fish (Fig. 1). Temperatures were recorded by a "Tinytalk" Datalogger. Everything was placed inside the polystyrene box which was covered with an outer cardboard carton (2 mm thickness). The parcel was dispatched by first class mail to the North Atlantic Fisheries College in Shetland (23 hr transit). From there it was immediately returned back to the FSL-Torry.

Mailprof computer program

The Mailprof computer program, Nesvadba (1992), was used for estimation of temperature changes of smoked salmon with gels (A, B, and C) or ice inside a polystyrene box. This program runs on a PC, and requires input values of surface heat transfer coefficient of the polystyrene box, thickness of fish, gels (or ice) and polystyrene box, initial temperature of fish and gels (or ice), freezing point of fish, mass of fish and gels (or ice), protein, fat and moisture content of the fish.

Proximate analyses of salmon

The total lipid content was determined by the Bligh and Dyer (1959) method as modified by Hanson and Olley (1963). The protein content was measured using an Automatic Gerhardt Analyser. Moisture content was determined by the European Economic Community recommended oven drying method - ISO 1442-1973 (Commission of European Communities, 1979).

Statistical analysis

Statistical significance was checked using Microsoft Excel Version 4 for Windows 3.1 Analysis Tools, Two Sample t-Test, assuming equal variances.

Table 2—Proximate analyses of salmon[a]

Samples	Moisture (%)	Protein (%)	Lipid (%)
Fresh salmon	75.14 ± 0.03	—	—
Smoked salmon	60.04 ± 0.2	—	—
Minced smoked salmon	59.60 ± 0.2	22.69 ± 0.11	16.21 ± 0.5

[a] Values are means of triplicate determinations

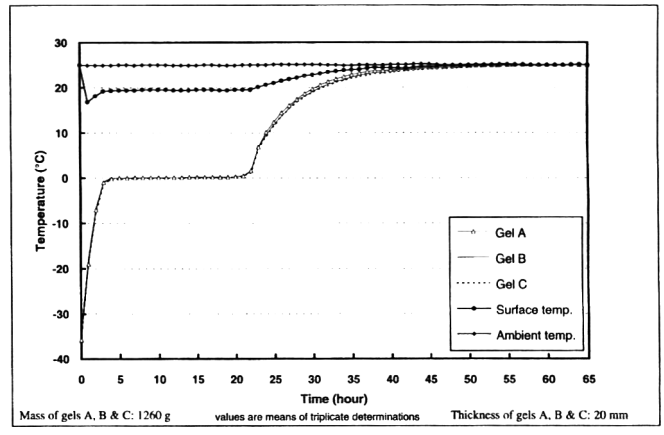

Fig. 3—Comparison of thawing of gels A, B and C at 25 ± 1°C inside a polystyrene box (20 mm thickness).

RESULTS & DISCUSSION

EXPERIMENTAL RESULTS for the latent heat capacity and thawing onset temperatures were compared (Table 1). No significant differences ($P > 0.05$) were found between the gels. The differences between ice and the gels could be explained by the gels containing hydrated organic co-polymers which could have decreased latent heat.

Compared to the DSC of fresh salmon (Fig. 2) the latent heat capacity was proportional to moisture content in smoked salmon. Although the fresh salmon contained 75.14 ± 0.03% moisture, the latent heat capacity indicated that a fraction of water in the fish was unfreezable. This was also found for smoked and minced smoked salmon (Tables 1 and 2).

Differences in thawing onset temperature were observed between fresh salmon, smoked salmon and minced smoked salmon (Table 1). The reason for these differences was considered to be the freezing point depression by salt. The salt content of smoked salmon may range from 3.6 to 8.1% (MAFF, 1991).

Time-temperature curves of the gels inside a polystyrene box were compared (Fig. 3 and 4). The ambient temperature was 25 ± 1°C. Thawing times were similar for all gels, whereas times were shorter when ice was used (Fig. 4). Further experiments confirmed that differences between gels and ice were due to ice movement inside the cooling packs. Subsequently partitioned packs (Fig. 1) were used to eliminate false temperature readings. The temperature of a pack without polystyrene (Fig. 4) showed thawing about 18.5–19 hr earlier and demonstrated the advantage of good thermal insulation.

The temperature changes in minced smoked salmon and surrounding polystyrene box (top and bottom) during packaging and shipping at ambient temperature were followed (Fig. 5). Surface temperatures varied from about 9.7 to 21.7°C throughout shipment. Gel A cooled the top surface of the box more than the bottom due to its position on top of the smoked salmon. The temperature in fish was <8°C for all times up to 23 hr. No temperature differences ($P > 0.05$) were found between duplicate records (middle and 20 mm ending from edge of fish) throughout shipment.

Temperature changes of minced smoked salmon, gel A and the surrounding polystyrene box at 25 ± 1°C were also com-

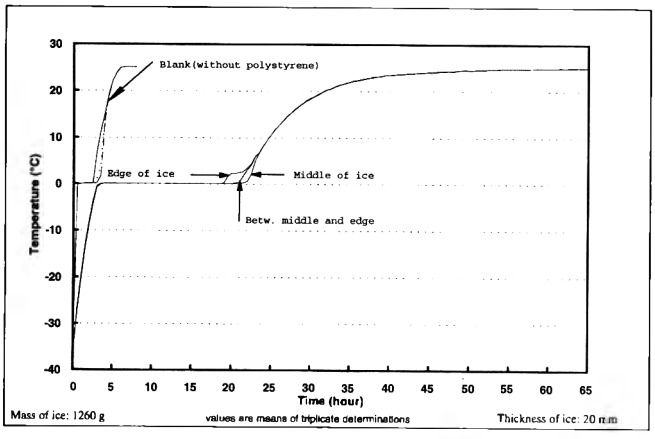

Fig. 4—Comparison of thawing of ice at 25 ± 1°C with and without polystyrene box.

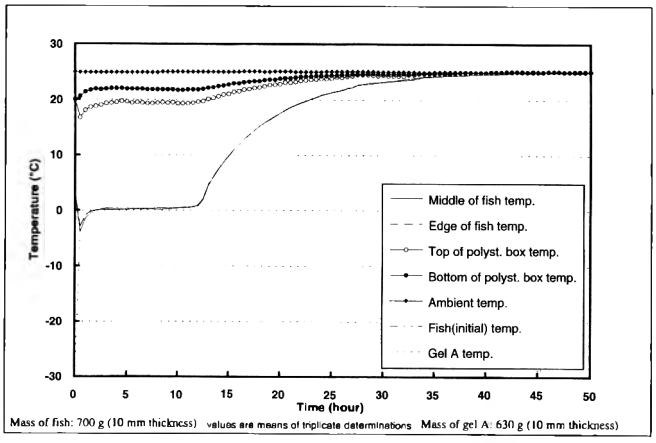

Fig. 6—Temperature changes of minced smoked salmon with gel A inside a polystyrene box at 25 ± 1°C.

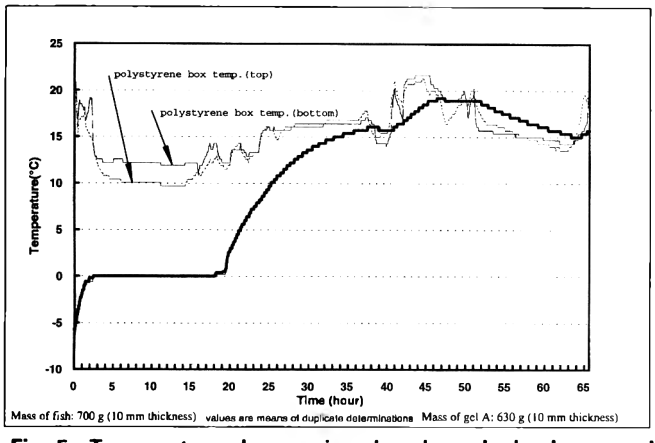

Fig. 5—Temperature changes in minced smoked salmon packaged with gel A inside a polystyrene box and shipped at ambient temperatures.

Table 3—Experimental data for Mailprof computer run[a]

PARAMETERS	Unit	Inputs
Water content of fish	%	59.60
Protein content of fish	%	22.69
Fat content of fish	%	16.21
Freezing point of fish	°C	−3.5
Water content of ice/gel	%	100
Freezing point of ice/gel	°C	0
Thickness of polystyrene box	mm	20
Mass of fish	kg	0.7
Mass of ice/gel	kg	0.63
Thickness of fish layer	mm	10
Thickness of ice/gel layer	mm	10
Initial fish temperature	°C	5
Initial ice/gel temperature	°C	−35
Surface temperature of box	°C	20
Surface heat transfer coefficient	W/m^2K	2.86

[a] These data were obtained from the experimental results except surface heat transfer coefficient, which was estimated by matching the predictions of the Mailprof computer program with experimental temperature records.

pared (Fig. 6). The surface temperatures of the polystyrene box (top and bottom) were lower than ambient temperature. These differences were due to the position of gel A in the box. Ice and gel A reached 8°C at the same time, although there were temperature differences between the middle and edge of fish during thawing when ice was used.

Some of the data were used for running the Mailprof computer program (Table 3). The temperature profile predicted by Mailprof (Fig. 7) agreed with experimental temperature curves for smoked salmon with gels A, B and C (or ice) (Fig. 6) in reaching 8°C at the same time. It was also possible to predict by computer modeling the temperature of a product in a time-temperature regime, provided the heat transfer coefficient of the packaging was known. Chill temperatures could be maintained for a predetermined time by using an optimum amount of gels (or ice) in relation to the degree of insulation and the cost of postage, calculated using the Mailprof computer program.

The results suggest that chill temperatures could be maintained for a predetermined time by using an amount of gels (or ice) calculated to be sufficient. The use of ice or gels helped maintain low temperatures during distribution. Note that the use of coolant does not *per se* ensure product safety. The time during which the product is at low temperature will depend on the mass and initial temperature of the product. The melting point of smoked salmon is affected by salt concentration which can show considerable variation.

Further work is needed to investigate the effects of salt concentration on latent heat of smoked salmon, the advantage of

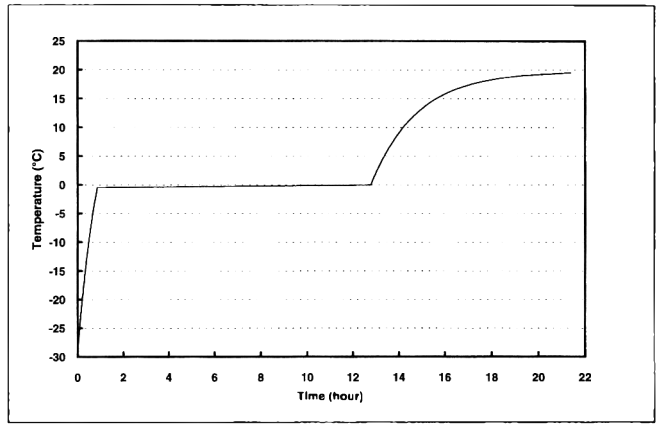

Fig. 7—Estimation of temperature changes of minced smoked salmon with gel A inside a polystyrene box stored at temperatures of 25 ± 1°C using Mailprof.

pre-freezing of products and the effects of distributing coolant both on top and bottom of products. It is also important to conduct studies of microbiological safety of smoked salmon. The probable numbers of organisms on these products could be estimated for each salt level and time-temperature history using predictive software such as the Food Micromodel (MAFF, 1994).

—Continued on page 626

Sardine Surimi Gels as Affected by Salt Concentration, Blending, Heat Treatment and Moisture

C. ALVAREZ, I. COUSO, and M. TEJADA

ABSTRACT

The effects of simultaneous modification of salt concentration, blending time, moisture content and heat treatment at different setting and cooking temperatures and time on characteristics of sardine (*Sardina pilchardus*) surimi gels was examined using a randomized incomplete block design. Maximum gel strength (GS) was obtained at highest salt concentrations and 78% moisture. Pre-setting was required to achieve acceptable gel quality. Highest GS values were found in gels set for 30–60 min at 35°C prior to heating at 90°C for 40 min. However, GS decreased after prolonged heating at 90°C. Gels set at 25, 35 and 40°C for 90 min had lower GS values when heated at 90°C for 40 min but were stable during further heating.

Key Words: sardine surimi, gel rheology, salt, blending, heat treatment.

INTRODUCTION

FOR PRODUCTION of thermally irreversible surimi hydrogels ("kamaboko" type gels), surimi must be ground with NaCl to solubilize actomyosin (AM), following which intermolecular bonds are established among proteins which are further stabilized by heating to form thermally irreversible gels. Texture varies with the species (Suzuki, 1981; Lanier et al., 1982; Kim et al., 1986), salt concentration (Sano, 1988), temperature and time at which the surimi is blended with salt (Lee and Toledo, 1976; Kato et al., 1989), gel moisture (Lee, 1984; Lanier, 1986; Alvarez et al., 1990; Hamann, 1990) and heat treatment (Okada, 1992). For some fish species the actomyosin sol can be set at 50°C, forming intermediate gels ("suwari" gels) with an elastic structure. This can be further heated (80–100°C) to form a firmer, more resistant structure. Surimi gels can also be made by direct heating with no pre-setting. The type and number of intermolecular bonds involved in formation of the different networks have been widely studied (Itoh et al., 1979; Ishiorosi et al., 1981; Sano, 1988; Beas et al., 1988; Roussel, 1988; Beas and Crupkin, 1990; Roussel and Cheftel, 1990; Niwa, 1992). Optimum gelation conditions have been established for surimi from several species but they appear to vary with species.

Sardine surimi sets very fast (Suzuki, 1981); however, reported study on gels made at different processing conditions is limited. The optimum setting temperature for *Sardina japonicus* was 30°C (Ishikawa, 1978) and for *Sardina pilchardus* 4°C and 37°C (Roussel, 1988). The difference between these results may have been due to the fact that the gels were made with different salt concentrations, blending times and final moisture. Therefore, interpretation of these results and their relation to the effects of any given parameter are not conclusive.

Our objective was to determine the most suitable salt concentration, moisture content, blending time and heat treatment for sardine surimi gels with maximum gel strength.

MATERIALS & METHODS

Experimental design

Four variables affecting final characteristics of gels (salt concentration, moisture content, blending time and heat treatment) were changed simultaneously. Three different levels were established on a surimi weight basis for salt concentration (2.5, 3 and 3.5g/100g surimi) ranging in gels from 1.88% to 2.8% according to moisture content of the gel, blending times (5, 7 and 10 min) and moisture contents (76, 78 and 80%) and different heating conditions (setting and cooking temperatures and times [25, 35 or 40°C and 30, 60 or 90 min and 90°C for 40 or 60 min]). The conditions or levels of variables for each lot were established using a model of incomplete randomized blocks resolved with a univariant analysis of variance for each response parameter. Each lot was prepared at least in triplicate.

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Materials

Frozen sardine (*Sardina pilchardus*) surimi prepared in one batch for this study by SCOMA (Lorient, France), was air-freighted with solid CO_2 to the laboratory, cut into blocks, vacuum-packed in Cryovac BB-1 bags (80 Torr pressure) and stored at -20 ± 1°C for 1 mo. Cryoprotectants added to the surimi were 4% sucrose, 4% sorbitol and 0.3% sodium tripolyphosphate. Two 10 kg blocks were used. Crude protein was measured by Kjeldahl method (AOAC, 1975), crude fat by Bligh and Dyer (1959) method as modified by Knudsen et al. (1985) and moisture and ash by AOAC (1975). The proximate composition of the surimi was: crude protein, 13.13%; crude fat, 3.05%; moisture, 75.82%; ash, 0.63% and pH 6.6.

Gel preparation

Surimi was tempered for about 2 hr at 21 ± 2°C until it reached -5 ± 1°C, then ground for 1 min (Stephan UM12 refrigerated vacuum cutter mixing machine [Stephan u Söhne GmbH & Co., Hameln, Germany], 0.1 bar, coolant temperature −2°C). Salt (NaCl) and ice-chilled water were added as necessary to adjust moisture content in each gel and the mixture was blended. Temperature of surimi sols was kept <10°C at all times (the temperature considered the limit beyond which temperature may negatively affect final texture, Babbitt and Reppond, 1988). The sols were heated in stainless steel cylinders (30 mm inner diameter × 30 mm height) with screw-on tops and bottoms, taking special care to ensure that sols were well packed and free of air bubbles. Sols were heat-set at 25, 35 or 40°C in a water bath (Julabo F10, Labortechnik GmbH, Seelbach, Germany) prior to cooking at 90°C for 40 or 60 min using a saturated steam oven (Rational Combi-Master CM6, Großküchentechnik GmbH, Landsberg a. Lech, Germany). Gels were also prepared by heating at 90°C for 40 or 60 min without pre-setting (direct cooking). After treatment, gels were cooled under running tap water and stored at 4 ± 1°C for 24 hr before gel analysis.

Rheological analysis

A penetration test was performed on samples (30 mm height × 30 mm diameter, 20 ± 1°C) using a cylindrical stainless-steel spherical probe (diam 5 mm) attached to a 100 N load cell connected to an Instron Universal Testing Machine model 4501 (Instron Engineering Corporation, Canton, MA.). A Hewlett-Packard Vectra ES/12 computer was used to program cross-head movement to 10 mm/min and to develop and analyze force-deformation curves. Gel strength (GS)(N.mm) was determined as the product of yield strength (YS)(N) and yield deformation (YD)(mm).

Water-holding capacity

Water-holding capacity (WHC) was determined following the method described by Roussel and Cheftel (1990) and modified by Alvarez et al. (1992). A 2-g sample (cut into about 3 mm cubes) was spun in a Sorvall RT6000B centrifuge at 3000 rpm for 15 min and the exudate collected

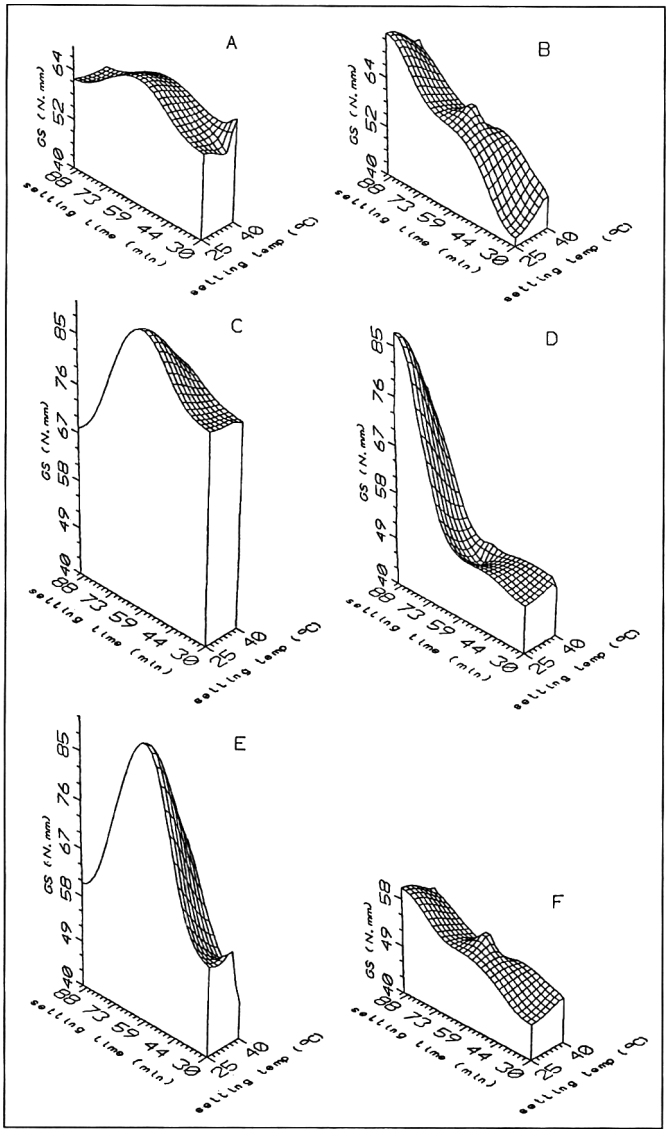

Fig. 1—Gel strength (N.mm) surface curves of sardine surimi gels prepared at different setting conditions: A, C and E, cooked 90°C for 40 min; B, D and F, cooked 90°C for 60 min. Moisture in A and B, 76%; C and D, 78%; E and F, 80%. Salt: A and B, 2.26%; C and D, 2.07%; E and F, 1.88% (protein/NaCl = 5.26).

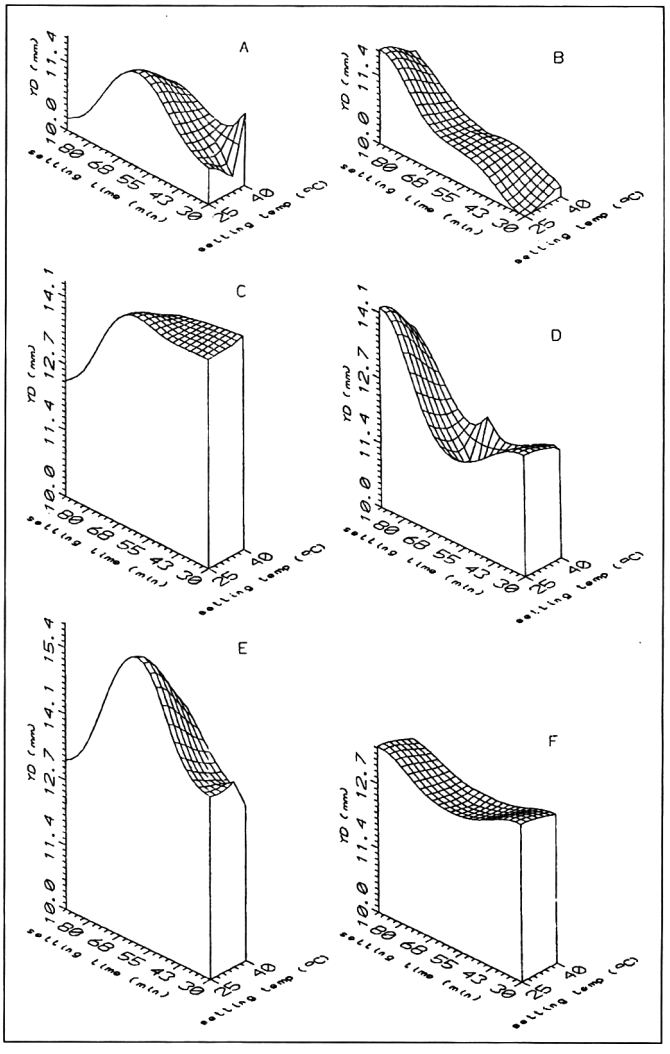

Fig. 2—Yield deformation (mm) surface curves of sardine surimi gels prepared at different setting conditions: A, C and E, cooked 90°C for 40 min; B, D and F, cooked 90°C for 60 min. Moisture in A and B, 76%; C and D, 78%; E and F, 80%. Salt: A and B, 2.26%; C and D, 2.07%; E and F, 1.88% (protein/NaCl = 5.26).

with Whatman filter paper. WHC was expressed as the percentage of water retained as related to the water present in the gel prior to centrifuging.

Statistical analysis

An analysis of variance was performed using the program of Statgraphics STSC Inc. (Rockville, MD). Unless stated, level of significance was set for $P < 0.05$.

RESULTS & DISCUSSION

Effect of moisture

GS values were compared (Fig. 1) for sardine surimi gels with different moisture contents, heat set, and cook conditions. The surimi was blended with 2.5% salt (protein/NaCl = 5.26) corresponding to NaCl in the gels of 2.26%, 2.07% and 1.88% for gels with 76%, 78% and 80% moisture. Highest GS values were obtained in gels with 78% moisture, although variable depending on setting and heating time and temperature. YD values were higher in gels with 78% and 80% moisture (Fig. 2) while YS was higher in gels with 76% and 78% moisture (Fig. 3). For all three moisture levels, YS fell sharply when gels were prepared by direct cooking, with the maximum of 2.66N in gels with 76% moisture and 60 min at 90°C. Generally YS increases with concentration of solids in the gel and YD values depend more on the intrinsic functicnal quality of the surimi (Lanier, 1986; Hamann, 1990). In these samples the gels were made from the same batch of surimi under identical solubilization and blending conditions, so we assumed that peak GS occurred because the gel matrixes formed under different thermal conditions were different. Maximum GS would not necessarily correspond to maximum values of solids concentration or total protein (Table 1).

As moisture decreased, a higher WHC resulted (Fig. 4), which might have come from increased solids in the gels. For gels with the same final moisture, the lowest WHC was observed when gels were directly cooked. This may have been because the rapid unfolding of proteins resulted in more intense coagulation and higher water release from those gels (Niwa, 1992). No significant differences in GS, YD or YS of gels were observed with different blending times (5, 7 or 10 min).

Effect of salt concentration

The GS in gels made with different NaCl concentrations were compared (Fig. 1C and D, 5A–D) as related to heat treatments and blending times (7 min). Highest GS values were observed with 2.44% and 2.8% NaCl and the lowest GS with 2.07% (Fig. 1). The reason for these results confirming other published re-

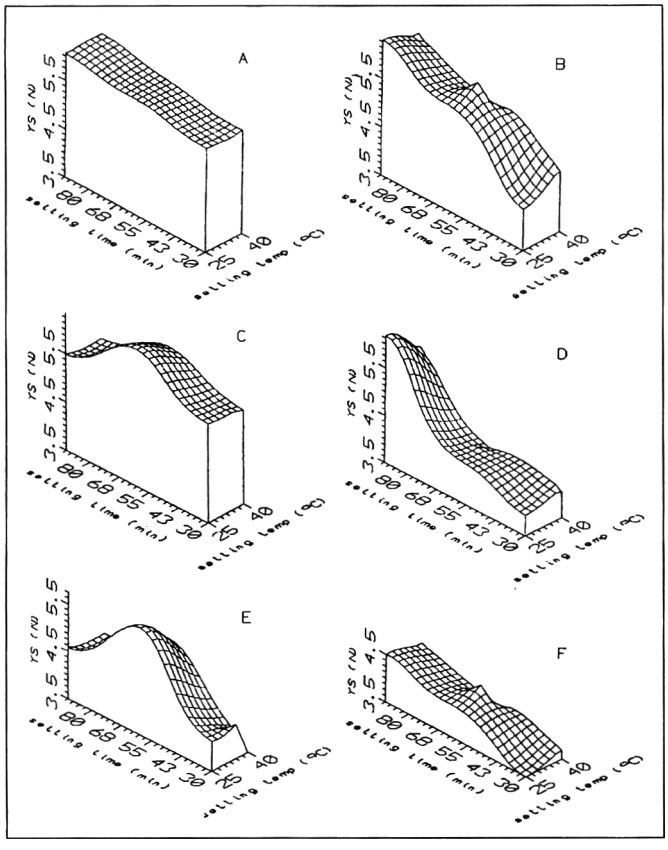

Fig. 3—Yield strength (N) surface curves of sardine surimi gels prepared at different setting conditions: A, C and E, cooked 90°C for 40 min; B, D and F, cooked 90°C for 60 min. Moisture content in A and B, 76%; C and D, 78%; E and F, 80%. Salt: A and B, 2.26%; C and D, 2.07%; E and F, 1.88% (protein/NaCl = 5.26).

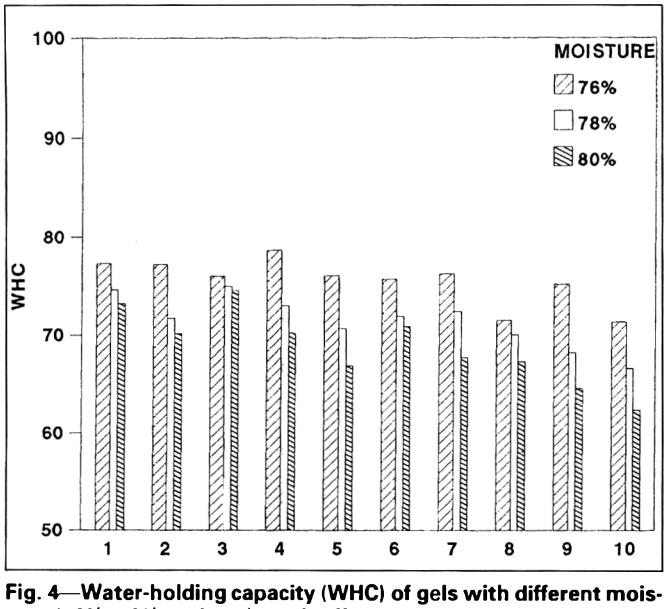

Fig. 4—Water-holding capacity (WHC) of gels with different moisture (76%, 78% and 80%) and different heat treatments: (1) 25°C 30 min, 90°C 60 min; (2) 25°C 60 min, 90°C 40 min; (3) 25°C 90 min, 90°C 60 min; (4) 35°C 30 min, 90°C 40 min; (5) 35°C 60 min, 90°C 60 min; (6) 35°C 90 min, 90°C 40 min; (7) 40°C 30 min, 90°C 40 min; (8) 40°C 60 min, 90°C 60 min; (9) 90°C 40 min; (10) 90°C 60 min (protein/NaCl = 5.26).

ports (Sano, 1988; Ito et al., 1990), may be that more salt soluble myofibrillar proteins were extracted at higher salt concentrations. YD (Fig. 6) and YS (Fig. 7) showed similar trends. They decreased in unset gels at all salt concentrations, with the max-

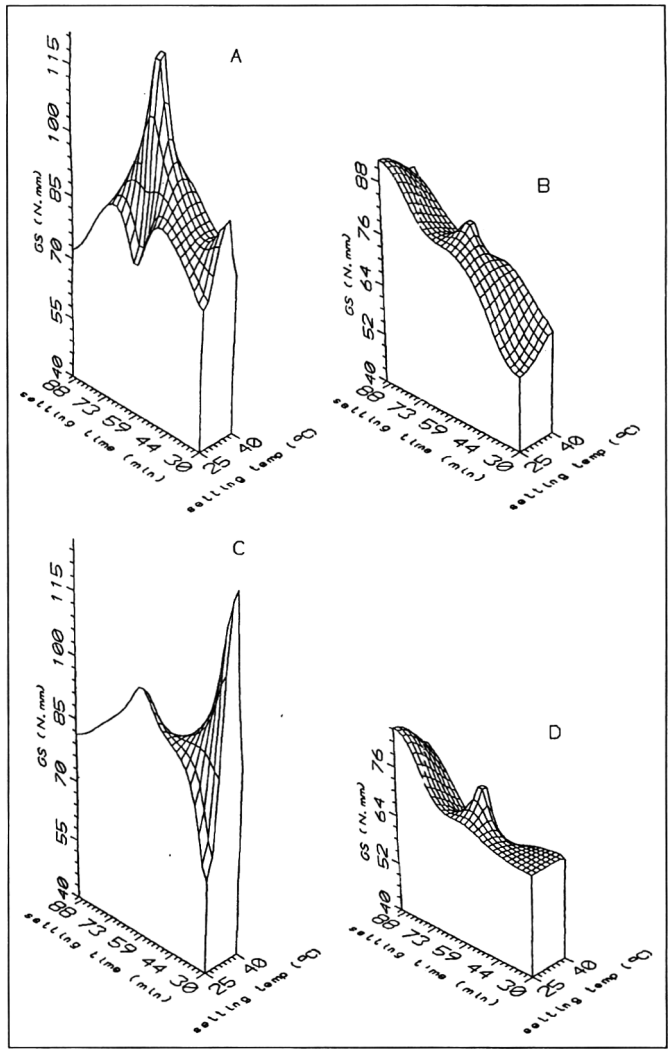

Fig. 5—Gel strength (N.mm) surface curves of sardine surimi gels prepared at different setting conditions: A and C, cooked 90°C for 40 min; B and D, cooked 90°C for 60 min. Moisture 78%. Salt: A and B, 2.44% (protein/NaCl = 4.38); C and D, 2.8% (protein/NaCl = 3.75)

Table 1—Protein and total solids as related to moisture content of gels

% Moisture	% Protein	% Total solids
76	11.89	24
78	10.69	22
80	9.90	20

imum for direct cooked gels 2.31N in gels prepared with 2.44% NaCl and 40 min direct cooking at 90°C. WHC showed an inverse relation to salt content (Fig. 8). The lowest WHC values ($p < 0.01$) were found in gels with 2.8% NaCl. This may have been related to competition between salt and proteins for the solvent, causing a smaller number of water molecules to interact with proteins and removal of more water and salt during centrifuging.

Effect of heat treatment

Heat setting was necessary to prepare sardine surimi gels of acceptable quality confirming other reports (Ishikawa, 1978; Suzuki, 1981; Roussel, 1988). In heat set gels, two trends were clearly observed depending on time of cooking at 90°C. When the pre-set gel was cooked at 90°C for 40 min (Fig. 1 A, C and E; Fig. 5 A and C), peak GS values occurred in gels set for 30 or 60 min but tended to decline in those where setting at any

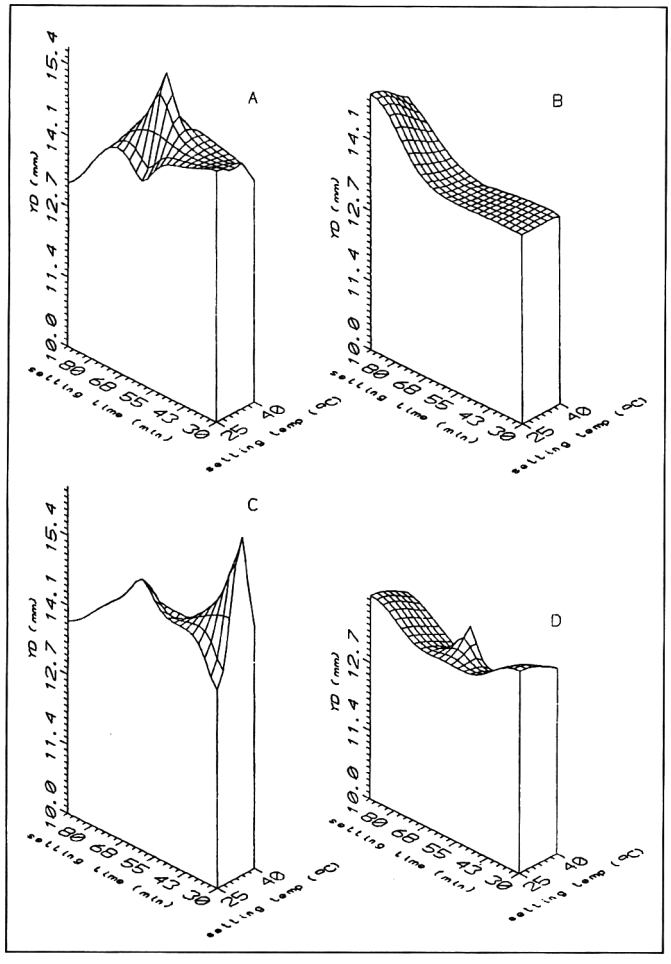

Fig. 6—Yield deformation (mm) surface curves of sardine surimi gels prepared at different setting conditions: A and C, cooked 90°C for 40 min; B and D, cooked 90°C for 60 min. Moisture 78%. Salt: A and B, 2.44% (protein/NaCl = 4.38); C and D, 2.8% (protein/NaCl = 3.75).

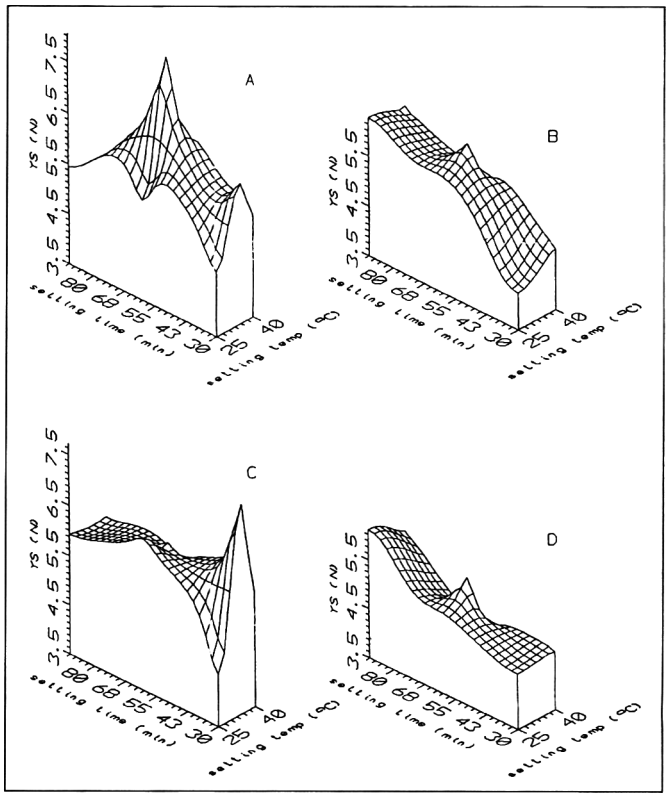

Fig. 7—Yield strength (N) surface curves of sardine surimi gels prepared at different setting conditions: A and C, cooked 90°C for 40 min; B and D, cooked 90°C for 60 min. Moisture 78%. Salt: A and B, 2.44% (protein/NaCl = 4.38); C and D, 2.8% (protein/NaCl = 3.75).

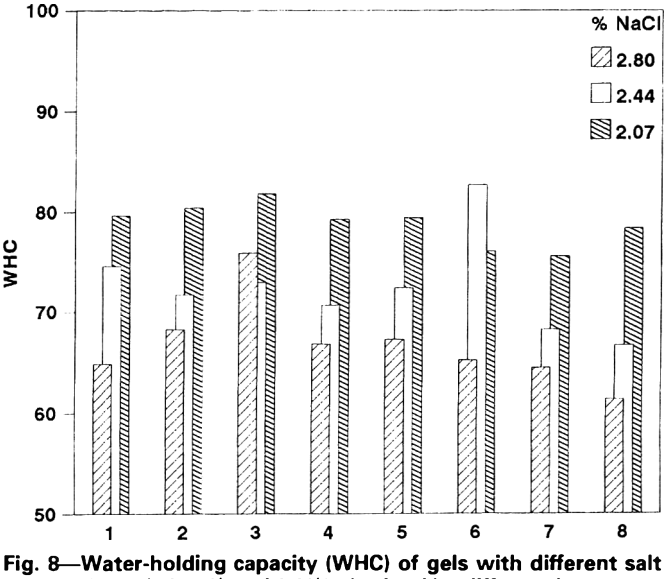

Fig. 8—Water-holding capacity (WHC) of gels with different salt content (2.07%, 2.44% and 2.8%) obtained by different heat treatments: (1) 25°C 30 min, 90°C 60 min; (2) 25°C 60 min, 90°C 40 min; (3) 35°C 30 min, 90°C 40 min; (4) 35°C 60 min, 90°C 60 min; (5) 40°C 30 min, 90°C 40 min; (6) 40°C 60 min, 90°C 60 min; (7) 90°C 40 min; (8) 90°C 60 min.

temperature had been prolonged beyond 60 min. However, when cooking at 90°C was prolonged up to 60 min, peak GS values occurred in gels that had been heat-set for 90 min (Fig. 1 B, D and F; Fig. 5 B and D), and were lower with shorter setting times.

Note that gels B, D (Fig. 1, 2, 3, 5, 6 and 7) and F (Fig. 1, 2 and 3), cooked for 60 min at 90°C passed through stages A, C (Fig. 1, 2, 3, 5, 6 and 7) and E (Fig. 1, 2 and 3), respectively, after 40 min cooking. This indicates that the networks formed by 30 or 60 min setting followed by 40 min cooking at 90°C were probably weakened by 20 min further cooking. The gels formed by 90 min setting, however were more stable to longer cooking (60 min) at 90°C, although at 40 min their GS was lower than in gels set for shorter periods. This was because in gels set for up to 60 min and cooked for 60 min at 90°C, both YD and YS were found to decrease but in those set for 90 min, YS remained stable. Thus, the slight increases in GS when 90 min set gels were cooked for 60 min were mainly due to an increase in YD. It therefore appears that in gels made with sardine surimi, the matrix networks formed during heat setting differed depending on setting time and temperature. This was probably due to the formation of different inter-molecular bonds between the proteins under differing heat conditions (Niwa, 1992). These different networks formed by setting, give rise to "kamaboko" gels with textures varying depending on how long they are subsequently cooked. For the formation of thermally irreversible gels, there appeared to be an optimum time for each setting-cooking combination. In some combinations, GS decreased if cooking was prolonged beyond a certain point. In sardine surimi gels, to obtain maximum GS, the moisture content of the gel and the amount of added salt must be taken into account, as both affect GS.

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Frozen Storage and Thawing Methods Affect Biochemical and Sensory Attributes of Rainbow Trout

KATARINA NILSSON and BO EKSTRAND

ABSTRACT

Rainbow trout were subjected to four thawing treatments after being stored at −18°C and −40°C for 0, 3, 7, 13 and 18 mo. Membrane integrity was estimated as the volume of centrifuged tissue fluid (CTF) and by lysosomal β-N-acetylglucosaminidase (NAG) activity in CTF. Slow thawing, in air at 5°C, resulted in higher NAG activity in CTF and a larger volume of CTF than fast thawing, at 25°C in water, independent of storage time. After 3 or more months storage, a higher NAG activity in CTF and a larger volume of CTF were found in all −18°C stored samples compared to that at −40°C. Sensory evaluation confirmed differences between trout stored at −18°C and at −40°C for 18 mo.

Key Words: trout, frozen storage, thaw temperatures, enzyme marker

INTRODUCTION

THE QUALITY OF FROZEN FISH depends, apart from the primary quality of the fresh fish, on the design of the freezing process, including storage and thawing. Fast freezing after rigor mortis is considered optimal for fish (Anonymous, 1972). Constant storage conditions are important and thawing also influences final quality. The limiting factor of frozen storage in lean fish is the aggregation of protein (Sikorski et al., 1976; Mackie, 1993), resulting in firmer fillets with low water-holding capacity. Lipid oxidation is the major limiting factor in frozen storage of fat fish (Hultin, 1992). However, salmon are very stable to lipid oxidation during frozen storage (Sörensen, 1993) although they are fat fish. This might be due to naturally occurring antioxidant mechanisms such as those reported in the cytosol of trout muscle (Han and Liston, 1989).

Freezing and thawing also affect the membrane structures of muscle cells (Fennema, 1978). The disintegration of membrane structures can be measured by the activity of one or more enzymes in muscle tissue fluids, enzymes that in fresh tissue are retained in intracellular organelles (Rehbein et al., 1978). The leaked enzymes are regarded as markers of membrane damage and the activity of lysosomal enzymes in the centrifuged tissue fluid has been used to differentiate frozen from fresh fish (Rehbein, 1979; Salfi et al., 1985; Shimomura et al., 1987). They have also been measured to estimate the effects of different freeze-thaw treatments (Nilsson and Ekstrand, 1993, 1994) on fish muscle structure.

Our objective was to determine the effects of frozen storage time and temperature, in combination with different thawing treatments, on membrane integrity of rainbow trout muscle based on a lysosomal marker enzyme. We also correlated objective enzyme measurements with sensory evaluations.

MATERIAL & METHODS

Fish

Farmed rainbow trout (*Oncorhynchus mykiss*) were obtained from Antens Trout Hatchery (near Göteborg, Sweden). The fish were killed by a blow to the head, bled, gutted and immediately put on ice for 3 days until resolution of rigor mortis. Body weight of eviscerated fish was 1030 g ± 160g.

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Freezing and frozen storage

Freezing was performed in a spiral belt freezer (GYRoFREEZE, Frigoscandia AB, Helsingborg, Sweden) at −35°C with air velocity 3.5 m/sec resulting in freezing time (3°C to −18°C in fish center) of 1.5 hr. Fish were then put in polyethylene bags and randomly distributed into groups for storage at −18°C and −40°C, for 0, 3, 7, 13, and 18 mo.

Thawing

Four different thawing treatments were applied to both the −18°C and the −40°C stored samples, varying thawing temperature and thawing medium (Table 1). Thawing time for each thaw-treatment group was defined as the time to increase the fish-center-temperature from storage temperature to 1°C. At 1°C thawing was defined as completed. Fish from the different storage groups started thawing with different internal temperatures (−18°C and −40°C). This difference stabilized during thawing at ~−5°C (Table 1).

Enzyme assay

For the preparation of CTF, slices (5–7g) of muscle were cut from the part of the fillet just in front of the dorsal fin. Skin, dorsal red muscle tissue and the fatty lower part of the belly flap were removed. Slices were centrifuged for 30 min at 28000 × *g*, and the fluid was collected with a Pasteur pipette for determination of volume, protein concentration and enzyme activity.

Homogenate (H) was prepared by disintegrating a muscle tissue slice (6 g), prepared as above, in 60 mL of 0.1M Tris-HCl buffer (pH 8.5) containing 5 mM EDTA and 0.2% Triton-X-100 in an Ultra Turrax T 25 (Janke and Kunkel, Staufen) at 2 × 30 sec at a speed of 13500 rpm. The mixture was kept on ice during homogenization. The homogenate was centrifuged as described. Enzyme activity and protein content of the supernatant fraction were determined.

The enzyme activity of β-N-acetylglucosaminidase (NAG) (E.C. 3.2.1.30) was measured spectrophotometrically using *p*-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate. The NAG activity in CTF from the homogenate represents specific activity from lysosomal leakage and that in the supernatant represents total enzyme activity within muscle tissue. The enzyme activity assay was described in detail by Nilsson and Ekstrand (1993).

The enzyme activity was calculated as katal (mole/sec) · g^{-1} protein. The total activity in each CTF and H sample was determined and related to tissue sample weight. To avoid large individual variations in absolute values of enzyme activity, the activity is given as the ratio (in percent) between enzyme activity in the CTF and activity in H.

The protein content was determined using the Coomassie Blue staining method according to Bradford (1976).

Peroxide value

Peroxide value was measured on fat extract from muscle slices similar to those used in the preparation of CTF. Fat extraction was by the

Table 1—Parameters and times of thawing treatments

Group	Media	Temp (°C)	Time[a] (hr)	Equal temp time[b] (hr)
I	Water bath	+25	0.8	0.20
II	Water bath	+5	4	0.35
III	Low circulating air	+25	7	4.40
IV	Low circulating air	+5	27	4.55

[a] Thawing time = time for fish center temperature to increase from storage temperature to 1°C.

[b] Time to reach equal fish center temperature during thawing for fish from different storage temperature groups.

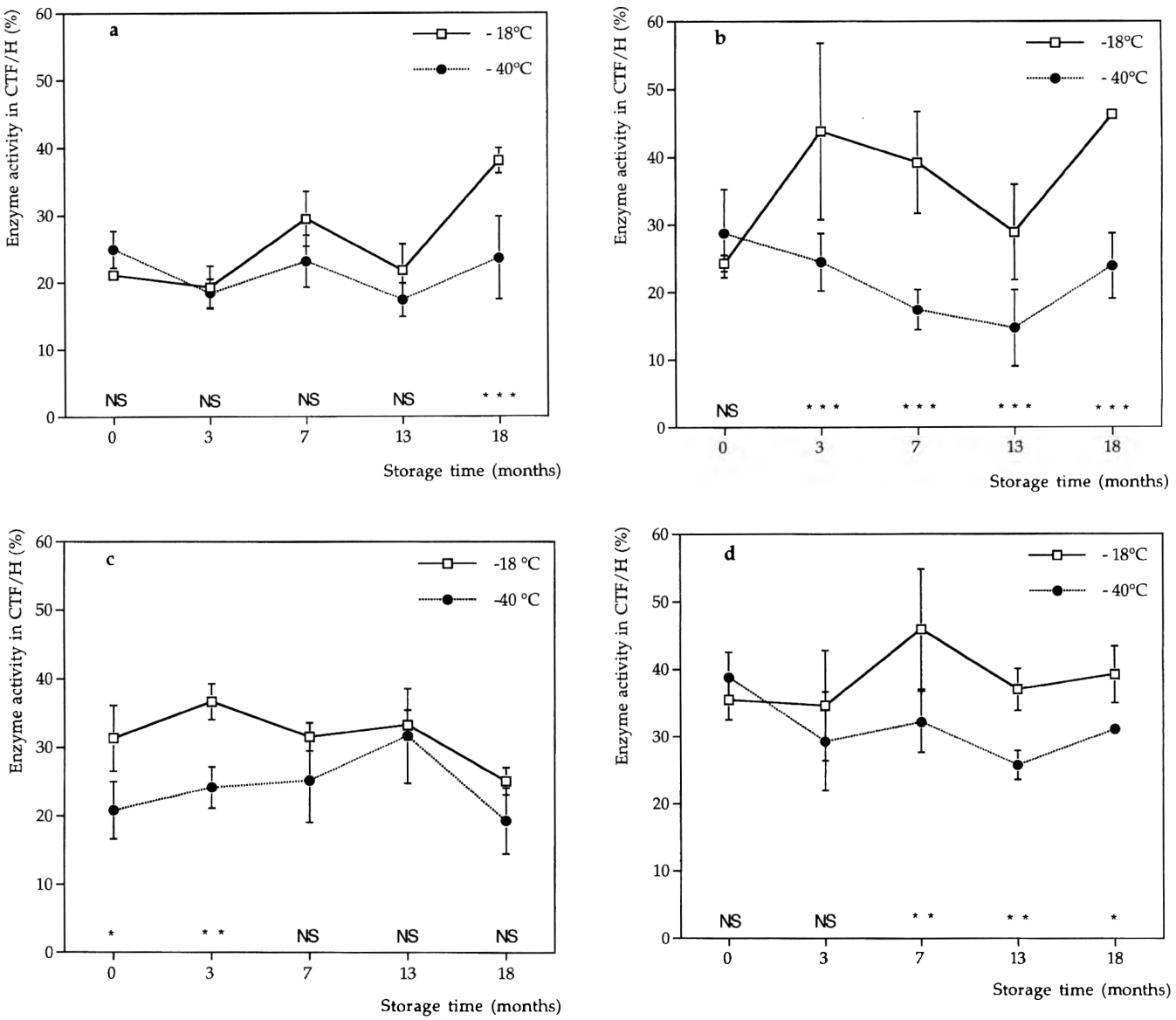

Fig. 1—β-N-acetylglucosaminidase (NAG) activity influenced by storage time and storage temperature after different thawing treatments: (a) in water 25°C; (b) in water 5°C; (c) in air 25°C; (d) in air 5°C. Enzyme activity of NAG expressed as % activity in CTF related to total enzyme activity measured in homogenate (H) from the same specimin. Means of three measurements of each n ± SD (n = 3). Standard deviation values ≤0.5 not shown. Statistical comparison of means from the two storage temperatures using the pooled standard deviation (4.93) from all 120 measurements. N.S = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

method of Bligh and Dyer (1959), and peroxide value was determined by the iodometric method (Cd-8-53, AOCS, 1990).

Sensory evaluation

After 18 mo of frozen storage, sensory evaluation focusing on attributes of "juiciness" and "firmness" was performed. Fish stored either at −18°C or −40°C were thawed in four different ways, as described. After thawing, the fish were filleted and adjusted to 5°C before heat treatment. The fillets were sealed in aluminum foil and cooked in a combi-oven (Combi-Matic, Elecrolux storkök AB, Sweden) using steam only. The oven temperature was 170°C and the heat treatment was interrupted when the sample center temperature reached 65°C (18.0 min ± 1.0 min). Four slices (60 g) were cut from the front part of each fillet, from the dorsal to the ventral side, and presented to panelists. Each panelist was given the same part of the different fillets tested. The panel consisted of eight persons familiar with sensory evaluations of fish. No specific training was done for this evaluation. At each session panel members were served four coded samples; −18°C and −40°C samples from two thawing groups, simultaneously, followed by a pause before the next taste session. The test was done in two replicates.

Definitions of attributes were discussed within the panel group before the first trial. Juiciness was defined as the perceived degree of liquid release during chewing, and firmness was defined as the perceived force required to compress the sample using the molar teeth. For intensity a continuous scale on a 100 mm line, anchored at the ends with the words "low" and "high" was used.

Statistical analysis

The project design was fully balanced and an analysis of variance (ANOVA) was performed on chemical and sensory data using SYSTAT (1992) computer package. For comparison of means, Student's t-test was used with a pooled standard deviation of all 120 data measurements for each chemical parameter.

RESULTS & DISCUSSION

Marker enzyme activity

Activity of NAG was measured in CTF as an index of membrane disintegration occurring in fish muscle due to frozen storage conditions and different thawing treatments (Nilsson and Ekstrand, 1993, 1994). To estimate the effects of thawing treatments with minimized storage, trout stored at a ≤10 days (0 month-samples) in −18°C and −40°C were used. A general

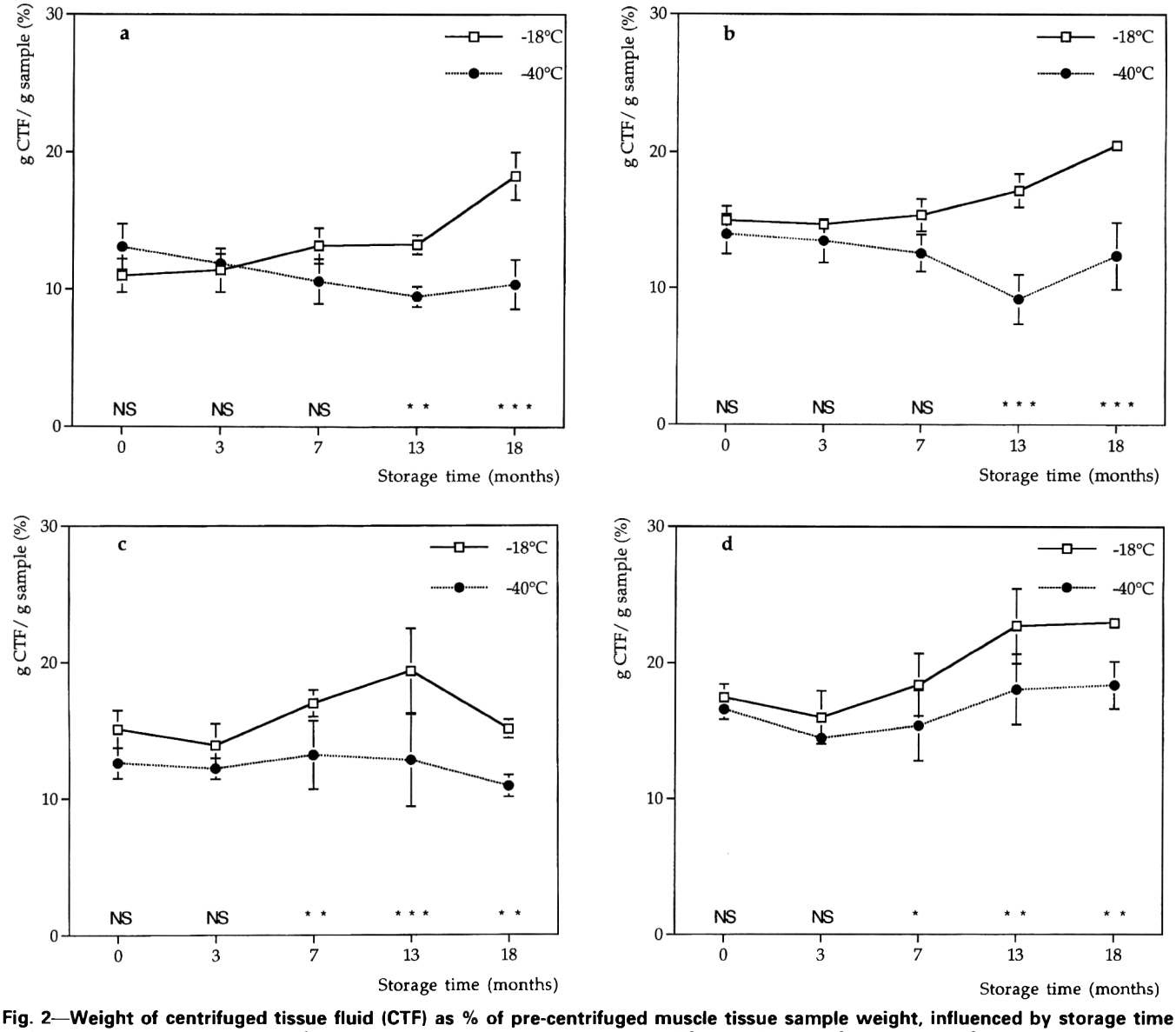

Fig. 2—Weight of centrifuged tissue fluid (CTF) as % of pre-centrifuged muscle tissue sample weight, influenced by storage time and storage temperature after different thawing treatments: (a) in water 25°C; (b) in water 5°C; (c) in air 25°C; (d) in air 5°C. Means of three measurements of each n ± SD (n = 3). Standard deviation values ≤ 0.5 not shown. Statistical comparison of means from the two storage temperatures using the pooled standard deviation (1.67) from all 120 measurements. N.S = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

increase in NAG activity at 0 months occurred with increasing thawing time for the −18°C samples (Fig. 1 a–d). The longest thawing time (5°C air) resulted in a higher NAG activity ($p < 0.05$) than the other three thawing groups in the fish stored at −40°C and higher NAG activity than the two fastest thawing groups in the fish stored at −18°C ($p < 0.05$).

Comparing the temperature groups at 3 mo storage, the fish at −18°C showed higher activity for 5°C water-thawed ($p < 0.001$) and 25°C air-thawed ($p < 0.01$) than fish stored at −40°C. This indicated that storage temperature had a marked effect on muscle membrane even after 3 mo storage. A tendency of higher enzyme activity in CTF in fish stored at −18°C compared to −40°C could be seen after 3 mo and throughout storage.

All values measured in the 5°C air-thawed samples (Fig. 1d) showed higher NAG activity than in the 25°C water-thawed sample (Fig. 1a), in both storage groups. As with earlier results (Nilsson and Ekstrand, 1994), this indicated that fast thawing influenced the membrane integrity less than slow thawing. Our results show this was independent of the length of frozen storage period. Thawing at 5°C (Fig. 1b) resulted in the largest differences in NAG activity between the 2 storage temperature groups ($p < 0.001$) at 3, 7, 13 and 18 mo storage.

By analysis of variance, storage temperature and thawing temperature resulted in major effects on NAG activity ($p < 0.001$). Although it had no significant major effect on NAG activity, storage time showed first-order interaction effects with both storage temperature ($p < 0.01$) and thawing media ($p < 0.001$) on NAG activity.

CTF

Effect of frozen storage on liquid loss (Fig. 2 a–d) were measured as volume of CTF after different thawing treatments. Trout stored at −18°C released more CTF than those stored at −40°C, seen after 7-mo storage (Fig. 2c and 2d) and after 13 mo storage (Fig. 2a and 2b). A tendency towards an increase in liquid loss in trout stored at −18°C was seen with increased storage time. However, in fish stored at −40°C, a decrease in CTF volume occurred with increasing storage time except for the slowest, 5°C air-thawed fish (Fig. 2d). In this thaw group, CTF increased in samples from both storage temperatures throughout the stor-

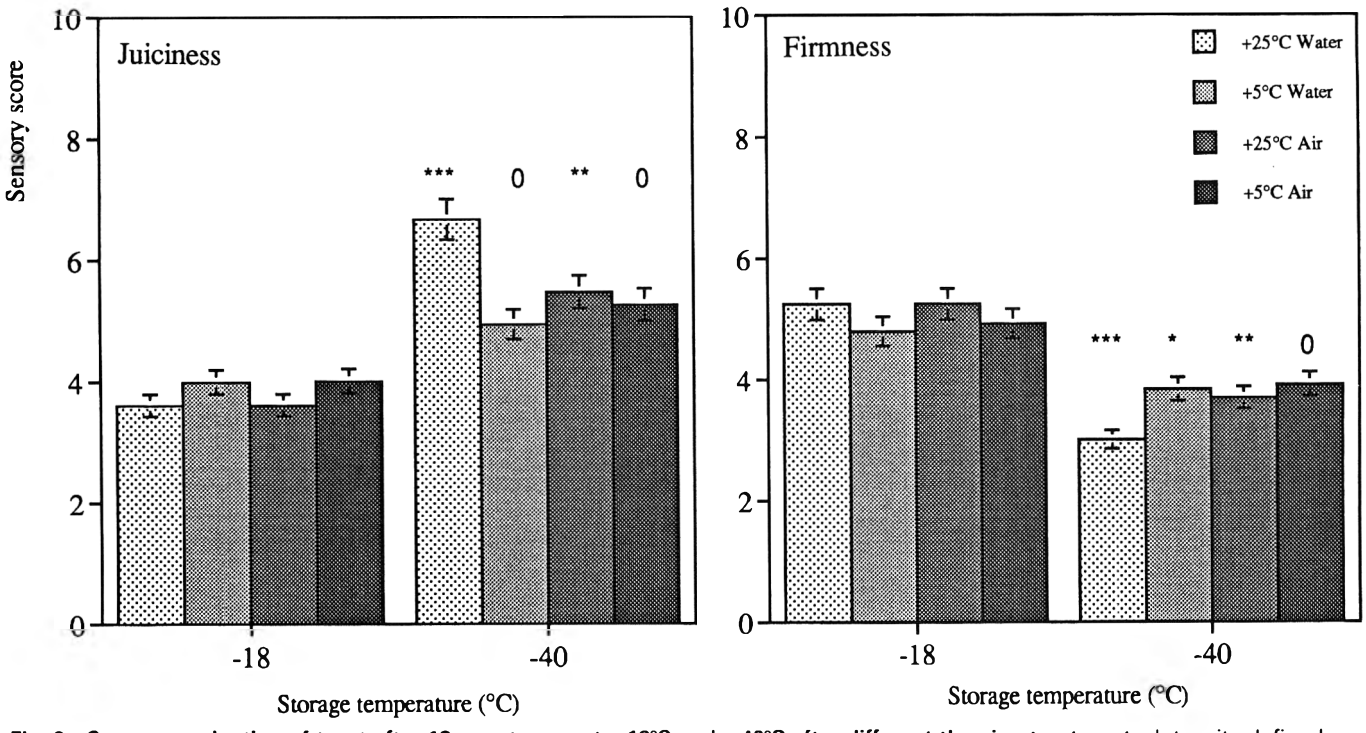

Fig. 3—Sensory evaluation of trout after 18 mo storage at −18°C and −40°C after different thawing treatments. Intensity defined on continuous scale on 100 mm line with words "low" and "high" anchored at ends. Means of 16 trials ± SE (n = 4). Statistical comparison of means between storage temperatures for the same thawing treatment. 0 = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

age period, and of the 4 thawing treatments, this group also showed the highest liquid loss. The lowest liquid loss occurred in the fastest thawing group, 25°C water (Fig. 2a).

Analysis of variance indicated a major effect ($p < 0.001$) for both storage time and temperature on CTF. A first-order interaction effect ($p < 0.001$) was also found. Effects of first-order interactions between thawing temperature-time of frozen storage ($p < 0.05$) and thawing media-time of frozen storage ($p < 0.001$) showed that the method of thawing had a marked influence on degree of liquid loss after frozen storage.

Both enzyme activity in CTF and release of CTF indicated that there were frozen storage effects on membrane structures. The overall effect was less after storage at −40°C than at −18°C. Differences in some thawing treatments between high and low storage temperature after 3 mos were more pronounced in enzyme activity measurements than in forced liquid loss CTF measurements. This indicated that enzyme measurements were more sensitive for estimating membrane disintegration than were forced liquid loss.

Sensory evaluation

After 18 mo storage, a major effect of storage temperature ($p < 0.001$) was seen on both juiciness (Fig. 3a) and firmness (Fig. 3b). Fish stored at −18°C were generally scored lower in juiciness and higher in hardness than those stored at −40°C but the −18°C samples showed no differences between thawing treatments. In the group stored at −40°C the fish thawed fastest, in air 25°C, scored higher in juiciness ($p < 0.05$) than those subjected to other treatments. Regarding juiciness a major effect of thawing temperature ($p < 0.05$) was seen and a first-order interaction effect of storage temperature and thawing temperature ($p < 0.001$) was found. Fast thawing resulted in lower ($p < 0.05$) firmness compared to fish thawed at 5°C in air. Firmness data also revealed a second-order interaction effect of storage temperature, thawing temperature and thawing media ($p < 0.05$).

Peroxide value

During storage peroxide values were low (<5) both in fish stored at −18°C and those stored at −40°C, and no differences occurred between either storage or thawing groups. An explanation of the low peroxide values, even after 18-mo storage, could be the low fat content (2.5–3.5%) of the muscle slices used to estimate peroxide value and NAG activity. When measuring the peroxide value after 18-mo storage in a part of the fish with a very high fat content (the belly flap), the peroxide value was 40. This indicated ongoing lipid oxidation but only in the areas of lipid deposits. These fat parts of the fish were not used for chemical measurements or sensory evaluation.

CONCLUSION

LOWER STORAGE TEMPERATURE (−40°C) did not affect membrane structures after different thawing treatments as much as higher storage temperatures (−18°C). In general faster thawing resulted in less membrane disintegration than slower thawing, after 3, 7 and 13 mo storage whether stored at −18°C or at −40°C. Sensory evaluation also showed a difference between storage groups, but only differentiated faster thawing in fish stored at −40°C. Together with the biochemical measurements, this indicated that fast thawing was more important and had a greater influence on final quality of fish stored at −40°C than of those stored at −18°C.

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Differentiation of Cultured and Wild Sturgeon (*Acipenser oxyrinchus desotoi*) Based on Fatty Acid Composition

I-C. CHEN, F.A. CHAPMAN, C-I WEI, K.M. PORTIER, and S.F. O'KEEFE

ABSTRACT

Sturgeon species have attracted interest for aquaculture due to high value of the flesh, caviar and wild stock depletion. Lipid was extracted from sturgeon muscle using the Bligh and Dyer procedure. Fatty acids from total lipid were methylated using boron-trifluoride in methanol. Fatty acid methyl esters were analyzed by gas chromatography as weight %. The fatty acid profiles of lipids were different between cultured and wild sturgeon. Wild sturgeon had higher levels of 16:0, 16:1ω7, 18:1ω9, 22:4ω6 and 22:5ω6. Cultured fish had higher levels of 18:2ω6, C20 and C22 monoenes, 20:5ω3 and 22:6ω3. Stepwise discriminant analysis (SDA) was used to develop a mathematical model to distinguish the two populations; the levels of 16:2ω6, 22:5ω6 and phytanic acid accurately identified the two fish populations.

Key Words: sturgeon, fish, fatty acid, differentiation

INTRODUCTION

THE GULF OF MEXICO STURGEON (*Acipenser oxyrinchus desotoi*, also known as Gulf sturgeon), a subspecies of Atlantic sturgeon, is distributed from the Mississippi River to Tampa Bay (Wooley and Crateau, 1985; Barkuloo, 1988). Sturgeon have been fished commercially worldwide due to the high value of their eggs, for making caviar, and their flesh, which is often smoked. The gelatin extracted from their swim bladder (isinglass) has been used in wine and beer clarification, jellies and glues. Because of damming, overfishing, habitat destruction, and water pollution, Gulf sturgeon are rarely found inland in Florida, except in the Suwannee River which maintained a commercial fishery until the mid 1980s. In 1991, the Gulf of Mexico sturgeon was listed as a threatened species (U.S. Fish and Wildlife Service).

Cultured and wild sturgeon (including Gulf of Mexico sturgeon) cannot be differentiated from their morphology alone. Sturgeon meats are almost always sold with skin characteristics removed. The ability to differentiate cultured and wild fish can help in protection of wild stocks. Methods for identification of cultured and wild sturgeon origin based on differences in fatty acid compositions have not been established. Jahncke et al. (1988; 1992) reported that linoleic (18:2ω6) acid level was useful to differentiate cultured or wild origin for several different fish species. The 18:2ω6 levels in cultured fish were much higher than in wild fish, 11.7% ± 0.73 and 3.3% ± 0.37, respectively (Jahncke et al., 1992). Xu et al. (1993) have shown that the type of dietary lipid could profoundly affect the fatty acid composition of white sturgeon (*Acipenser transmontanus*) in culture.

Applications of discrimination procedures using fatty acid profiles of fish are rare. Knutsen et al. (1985) applied a pattern recognition algorithm (SIMCA, soft independent modeling of class analogy) to distinguish between cod and haddock eggs based on fatty acid profiles. Reportedly, that procedure was more effective than traditional discrimination techniques based on averages of profile values in each class (Blomquist et al., 1979). Our objective was to determine whether classical discrimination techniques were useful in separating wild from cultured sturgeon populations.

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MATERIALS & METHODS

Wild Gulf sturgeon were captured by gillnet from the Suwannee River during the Spring of 1992 and 1993. Captured fish were maintained in a holding tank with the oxygen content near saturation, for no more than 4 days, then transported live to the hatchery laboratories of the U.S. Fish and Wildlife Service (Gainesville, FL). Thirteen wild sturgeon were collected for this study. Cultured Gulf sturgeon were obtained from the Fisheries and Aquatic Sciences Department at the University of Florida. Female and male wild Gulf sturgeon captured in 1991 and 1992 were used as brood fish. These fish were injected with a carp pituitary extract to induce ovulation and spermination (Parauka et al., 1991). After ovulation and spermination, eggs were fertilized and incubated artifically using techniques developed for the white sturgeon (Doroshov et al., 1983). Larvae were fed on brine shrimp and chopped black worms for 5 wks, then the fish were put on a commercial salmon diet (BioDiet; Bioproducts, Inc., Warrenton, OR) over a 3-day transition period (1st day 1:4, 2nd 1:1, and 3rd 4:1; formulated: live diet, respectively). Ten 8-month-old and two 2-year-old cultured sturgeon were used.

Fatty acid composition

A cross-section of fish 5 cm thick was removed for each analysis from just behind the dorsal fin. Fat was extracted from the sections using the Bligh and Dyer (1959) method. Total lipids were transmethylated to fatty acid methyl esters with BF_3-methanol (Morrison and Smith, 1964). Fatty acids were analyzed using gas-liquid chromatography with a Shimadzu GC-14 gas chromatograph equipped with a split/splitless injector and a flame ionization detector (Shimadzu Scientific Instruments, Norcross, GA) and operated using He carrier gas at a linear flow ≈25 cm/sec and split pressure of 1.0 kg/cc (about 40:1). The column was an Omegawax™ 250 fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm; Supelco, Bellefonte, PA). Oven temperature was 165°C for 12 min, programmed to 185°C at 2°C/min, held for 5 min, then programmed to 235°C at 2°C/min, then held for 20 min. Fatty acid methyl esters were identified by comparison of retention times and equivalent chain length values under isothermal conditions with standards (Ackman, 1986).

Statistical analysis

Using 12 cultured and 13 wild Gulf sturgeon, 52 fatty acid components were measured and nine combination measurements were taken: saturated acids, monoenes, dienes, trienes, tetraenes, pentaenes, ω3 fatty acids, ω6 fatty acids, and ω3 to ω6 ratio. Initial data analysis included graphical examination for outliers. Due to a large number of "independent" variables and the relatively small number of observations, the stepwise discriminate analysis (Mclachlan, 1992) applying the StepDisc (SAS Institute Inc, 1989, Cary, NC) was used to screen out possible discriminators (fatty acids useful for classification). The following equation was used to determine to which population an observation applied:

$$D_j^2(X) = \hat{\mu}_j + \sum_{j=1}^{3} \hat{\beta}_{ij} \hat{\chi}_i$$

The μ_i and β_{ij} are constants (see Table 3), and are measurements of selected fatty acids. The j was the population of interest (j = 1 for cultured, 2 for wild). The probability of a new sample belonging to the population j was determined by the equation:

$$P(j|X) = [e^{-D_j^2(x)}] \div [\sum_{k=1}^{2} e^{-D_k^2(x)}]$$

The model was validated by crossvalidation-based misclassification estimates.

RESULTS & DISCUSSION

Fatty acid compositions of total lipids

Several reviews have been published on fatty acid compositions of fish (Hearn et al., 1987; Ackman and Mcleod, 1988; Armstrong et al., 1991), but information for sturgeon species found in the U.S. is very limited. Ackman et al. (1972) reported the fatty acid composition of two specimens of Atlantic sturgeon and data for Gulf sturgeon are unavailable. The fatty acid compositions of cultured Gulf sturgeon ranged from 24.5–30.1% saturated, 31.6–47.2% monoenes, 2.6–9.3% dienes, 0.8–1.9% trienes, 2.6–5.4% tetraenes, 6.6–14.3% pentaenes, and 5.6–15.9% hexaenes (Table 1). Wild Gulf sturgeon had about 30.3–33.5% saturated acids, 46.8–54.2% monoenes, and the remainder were polyunsaturated acids.

The 16:0 contributed >60% of saturated fatty acids, and was therefore primarily responsible for the higher level of saturated fatty acids in wild Gulf sturgeon. The 14:0 and most other saturated fatty acid levels were higher in cultured than in wild fish. Mugrditchian et al. (1981) and Yu et al. (1977) reported that 16:0 and 18:0 were biosynthesized by fish to keep flesh saturated fatty acid levels constant. Thus, the higher amount of 18:0 in cultured Gulf sturgeon might be used to compensate for the otherwise low content of saturated fatty acids. Note that the percentages of 14:0 and 18:0 from lipids of older cultured Gulf sturgeon were higher than for younger fish, suggesting that some difference may be due to maturity or age. The small number of older cultured Gulf sturgeon maintained in culture precluded more extensive sampling.

The amount of total monoenes was higher in wild fish than in cultured. The contents of some monoenes, such as 16:1ω9, 16:1ω7, and 18:1ω9, were higher in wild fish, while that of 18:1ω7 and some long-chain monoenes, such as 20:1ω11, 20:1ω9, 22:1ω11 and 22:1ω9, were higher in cultured sturgeon. The patterns of monoene composition of cultured Gulf sturgeon were similar to their feed fatty acid compositions. Although information on diet composition was unavailable from the manufacturer, it is likely that herring or menhaden meal was used in the feed (based on fatty acid composition and popularity of those fishmeals). This would explain the long-chain monoene concentrations, as well as the higher levels of long-chain ω3 fatty acids in cultured fish.

Except for amounts of 18:2ω6 and its metabolic product, 20:4ω6 (arachidonic acid; AA), percentages of most dienes, trienes, tetraenes were <1.0%. The level of 18:2ω6 was greater in cultured than in wild sturgeon, in agreement with data from other fish species (Jahncke et al., 1988, 1992). Higher levels of dienes and ω6 fatty acids in cultured fish were largely the result of a much higher 18:2ω6 content. The level of 16:2ω6 was higher in wild than cultured fish (Fig. 1). Cultured and wild Gulf sturgeon had average AA contents of 2.5% and 1.4%, respectively. These levels were lower than published data for most northern and southern hemisphere fish (averages 4.7% and 3.2%, respectively, Armstrong et al., 1991). Since AA may have effects antagonistic to the health benefits of the ω3 fatty acids, relatively lower amounts of AA might be advantageous to the consumer for cardiovascular health (Kinsella, 1986; Sanders, 1986) in the Western diet, which is relatively low in ω3 fatty acids. In contrast to 18:2ω6, the long-chain ω6 highly unsaturated fatty acids (HUFA), such as 22:4ω6 and 22:5ω6, were higher in wild sturgeon than in cultured. The ranges of these two fatty acids did not overlap between cultured and wild sturgeon, suggesting that they may be useful in discriminating between the populations. Higher levels of long-chain ω6 fatty acids (20:4ω6, 22:4ω6, 22:5ω6) and lower levels of 18:2ω6 were reported in wild Atlantic salmon smolt compared to cultured fish (Ackman and Takeuchi, 1986). This suggested that the elongation and desaturation of the 18:2ω6, amply provided in the cultured fish diet, was limited due to high levels of dietary long-chain ω3 fatty acids. The differences in levels of eicosanoid precursor fatty acids in these fish may have affected fish health. The long-chain ω6 fatty acids decreased and ω3 increased during development of sturgeon from fertilized egg to feeding larvae (Gershanovich, 1991). Higher levels of ω3 fatty acids in phospholipid (PL) of Atlantic sturgeon were reported in wild compared to cultured fish (Ackman and Takeuchi, 1986).

Among the ω3 series fatty acids, the levels of 18:3ω3, 18:4ω3, 20:4ω3 and 22:5ω3 seemed very similar among the sturgeon groups. However, the cultured fish had >four times more 20:5ω3 than the wild fish (Fig. 2). Since high amounts of this fatty acid appeared in lipids of the fish feed, the higher amount of 20:5ω3 in cultured Gulf sturgeon must have come from the formulated diet, and been deposited in flesh lipids (Pigott, 1989). The ω3 fatty acid compositions for cultured Gulf sturgeon and fish feed were similar to those reported by Kennish et al. (1992) for three salmon stocks fed a commercial diet, Biodiet. The percentage of 22:6ω3 in cultured Gulf sturgeon was higher than that of wild fish, and also higher than their diets. Since the lipid content of fish feed was higher than muscle lipid of cultured fish, the accumulation of this fatty acid in cultured fish may come from selective retention of the 22:6ω3 or from conversion of 20:5ω3 to 22:6ω3.

EPA (20:5ω3) is the most important essential fatty acid of the ω3 class in the human diet, since it is the precursor to the 3-series eicosanoids. They competitively inhibit platelet aggregation and increase production of anti-aggregatory agents (Holub, 1992). Several studies mentioned that DHA (22:6ω3) and EPA were interchangeable by retrogradation (Ackman and Burgher, 1965; von Schacky and Weber, 1985). In addition, DHA has been known to decrease the concentration of low-density lipoprotein cholesterol (LDL-C) in plasma (Childs et al., 1990). Since EPA and DHA were higher in cultured fish, the value of cultured sturgeon as a source of those fatty acids would be increased, in contrast with other fish species grown in culture (Pigott, 1989).

Statistical discrimination of fish populations

Wide differences were found in levels of fatty acids 18:2ω6, 16:2ω6, phytanic acid and 22:5ω6. The differences in 16:2ω6 levels (Fig. 1) were normally distributed and well separated. For 18:2ω6, the separation was also quite large; however, the distribution in cultured sturgeon was unexpected. The four data points above 2.6% were far from the other data, which were clustered in the 1.4–2.6% range. This unusual data spread resulted in higher variance, diminishing the usefulness of this fatty acid for discrimination.

The partial R^2 and average squared canonical correlation (ASCC) values were used to evaluate the relative importance of each variable. As more variables were included in the linear discriminant function, the partial R^2 values decreased and the ASCC, a measure of how well the populations are separated, approached 1. Ten (10) fatty acid measurements were found most applicable for discriminating between cultured and wild sturgeon (Table 2). The most useful fatty acid measurement for discriminating was 16:2ω6 (ASCC = 0.944). Adding the next two most differentiating, 22:5ω6 and phytanic acid, to the model increased the ASCC by 0.045, to 0.989. These two populations were readily distinguishable (Fig. 3) based on these 3 variables. A model with these three fatty acids was found to be dependable using misclassification estimates obtained with cross-validation techniques.

Relevance of linear discriminant model

Differences in fatty acid composition most likely reflect differences in diets between cultured and wild fish, although an

Table 1—Fatty acid compositions of cultured and wild Gulf of Mexico sturgeon and feed[a]

	Cultured					Wild			
FA	Min	Max	Avg	SD	Feed	Min	Max	Avg	SD
12:0	0.01	0.05	0.03	0.01	0.11	0.03	0.13	0.06	0.03
14:0	1.10	4.02	2.20	0.91	5.15	1.06	1.91	1.43	0.29
I-15:0	0.05	0.15	0.09	0.03	0.18	0.15	0.27	0.20	0.04
AI-15:0	0.03	0.08	0.04	0.01	0.03	0.04	0.07	0.05	0.01
15:0	0.20	0.38	0.26	0.06	0.41	0.30	0.53	0.38	0.07
I-16:0	0.00	0.07	0.03	0.02	0.04	0.14	0.22	0.18	0.02
16:0	15.3	20.1	17.6	1.64	19.2	24.1	27.2	25.9	0.84
Me-16:0	0.06	0.14	0.11	0.02	0.18	0.06	0.10	0.08	0.02
Phytanic	0.16	0.25	0.20	0.02	0.19	0.05	0.16	0.12	0.03
17:0	0.30	0.64	0.40	0.10	0.97	0.54	0.69	0.60	0.05
18:0	2.24	8.64	5.72	1.76	3.85	1.82	3.72	2.61	0.46
20:0	0.09	0.25	0.13	0.05	0.21	0.08	0.19	0.15	0.03
Saturated	24.5	30.1	26.8	2.19	30.5	30.3	33.5	31.8	1.02
16:1ω9	0.26	0.35	0.29	0.03	0.24	0.38	0.74	0.55	0.10
16:1ω7	2.16	5.98	3.33	1.20	7.40	7.19	10.6	8.92	0.99
16:1ω5	0.11	0.29	0.20	0.05	0.26	0.23	0.39	0.31	0.06
17:1ω8	0.13	0.37	0.26	0.07	0.32	0.64	0.86	0.72	0.06
18:1ω9	19.0	29.1	24.3	3.00	20.4	33.4	37.8	35.7	1.24
18:1ω7	4.40	5.23	4.84	0.36	4.43	2.38	3.60	2.74	0.35
18:1ω5	0.18	0.31	0.25	0.04	0.25	0.10	0.33	0.15	0.06
20:1ω11	1.11	3.49	2.58	0.82	1.66	0.15	1.16	0.35	0.27
20:1ω9	1.52	2.82	2.45	0.38	2.47	0.90	1.19	1.11	0.10
20:1ω7	0.20	0.45	0.36	0.08	0.44	0.16	1.21	0.33	0.28
22:1ω11	0.49	1.64	1.12	0.36	3.19	0.01	0.05	0.02	0.01
22:1ω9	0.37	0.83	0.56	0.12	0.53	0.09	0.14	0.10	0.01
22:1ω7	0.04	0.09	0.06	0.01	0.14	0.01	0.11	0.04	0.03
Monounsat	31.6	47.2	40.5	5.14	41.8	46.8	54.2	51.0	1.76
16:2ω6	0.07	0.12	0.10	0.02	0.10	0.22	0.29	0.26	0.02
16:2ω4	0.17	0.40	0.25	0.07	0.73	0.08	0.19	0.12	0.03
18:2ω9	0.03	0.09	0.07	0.02	0.04	0.32	0.76	0.45	0.12
18:2ω7	0.05	0.10	0.07	0.02	0.03	0.03	0.08	0.05	0.01
18:2ω6	1.63	7.98	3.25	1.94	2.43	0.35	0.51	0.40	0.05
18:2ω4	0.16	0.24	0.18	0.02	0.23	0.04	0.20	0.12	0.06
20:2nmid	0.03	0.32	0.11	0.09	0.02	0.13	0.29	0.17	0.05
20:2ω6	0.31	0.66	0.38	0.10	0.18	0.08	0.18	0.12	0.03
Diene	2.60	9.30	4.41	2.01	3.77	1.33	2.30	1.70	0.24
16:3ω4	0.05	0.28	0.12	0.07	0.55	0.02	0.14	0.08	0.04
18:3ω6	0.05	0.14	0.08	0.03	0.10	0.06	0.35	0.16	0.11
18:3ω4	0.18	0.30	0.24	0.04	0.10	0.10	0.28	0.16	0.05
18:3ω3	0.20	0.88	0.38	0.20	0.59	0.14	0.33	0.26	0.05
20:3ω6	0.16	0.22	0.18	0.02	0.10	0.11	0.20	0.15	0.03
20:3ω3	0.05	0.12	0.09	0.02	0.03	0.04	0.07	0.06	0.01
Triene	0.79	1.88	1.09	0.31	1.47	0.72	1.04	0.86	0.12
16:4ω3	0.00	0.11	0.03	0.03	0.02	0.04	0.13	0.07	0.02
16:4ω1	0.07	0.54	0.18	0.13	0.86	0.03	0.10	0.06	0.02
18:4ω3	0.20	0.69	0.36	0.14	1.19	0.16	0.40	0.31	0.06
18:4ω1	0.08	0.25	0.13	0.06	0.14	0.01	0.09	0.04	0.02
20:4ω6	0.98	4.22	2.50	1.01	0.83	0.61	2.72	1.36	0.55
20:4ω3	0.38	0.77	0.53	0.11	0.47	0.29	0.53	0.42	0.06
22:4ω6	0.13	0.39	0.27	0.10	0.08	0.78	1.24	0.97	0.12
22:4ω3	0.02	0.13	0.06	0.03	0.00	0.03	0.04	0.04	0.00
Tetraene	2.55	5.40	4.04	0.82	3.59	2.36	4.63	3.26	0.56
20:5ω3	4.64	11.8	8.28	1.95	9.97	1.07	3.11	1.78	0.71
21:5ω3	0.09	0.30	0.17	0.06	0.36	0.07	0.17	0.11	0.03
22:5ω6	0.17	0.34	0.26	0.05	0.12	0.68	1.57	1.23	0.24
22:5ω3	1.65	2.77	2.21	0.31	1.30	1.82	2.49	2.18	0.21
Pentaene	6.63	14.3	10.9	2.10	11.8	4.56	7.06	5.30	0.75
22:6ω3	5.58	15.9	12.2	3.41	7.16	2.71	8.85	6.10	1.34
Σω3	13.8	30.2	24.3	4.95	20.5	8.65	15.4	11.3	1.56
Σω6	5.91	10.0	7.02	1.16	3.95	3.54	6.29	4.64	0.79
Σω3/Σω6	1.66	4.24	3.58	0.92	5.19	1.82	3.36	2.48	0.41

[a] Abbreviations: FA—fatty acid; Avg.—average; SD—standard deviation; I—iso; AI—anteiso; Me—methyl; nmid—nonmethylene interrupted diene; monounsat—monounsaturated.

effect of age may also apply. Use of fatty acid composition for origin discrimination, therefore, relies on dietary differences as well as a clear understanding of the variability expected. Changing diets of cultured sturgeon would result in differences in fatty acid composition (Xu et al., 1993), and most likely affect the selection of fatty acids as discriminators. As well, if a diet supplier changed the composition of the diet, to change levels or eliminate fishmeal or to use a fishmeal with considerably different fatty acid composition, for example, new models would be required. There is no control over the diets of the wild fish. Shifting patterns of fatty acid composition could result from variations in food types related to pollution, climactic changes, alterations in migration patterns, etc. Therefore, for the discriminant model to be applicable, it is necessary to continuously monitor fatty acid compositions of wild and cultured fish to ensure that large changes do not occur and to continuously adjust the discriminant model functions as necessary.

Two of the three fatty acids in the linear discriminant model were from the ω6 fatty acid classes. Because fish can interconvert fatty acids in the ω6 class by elongation and desaturation, it was informative to examine possible origins of the differences. It is probably overly simplistic to suggest that differences were completely diet-related and metabolism was not involved. The ratios of average levels in cultured and wild fish for fatty acids of the ω3 and ω6 classes (Fig. 2) showed some obvious differences in relative levels of fatty acids within one family class (ω6 or ω3). For example, among the ω6 fatty acids, the 18:2ω6 was much higher in cultured and wild. As the chain length increased and unsaturation increased, the relative levels decreased up until 22:4w6 and 22:5w6, where the wild fish had much

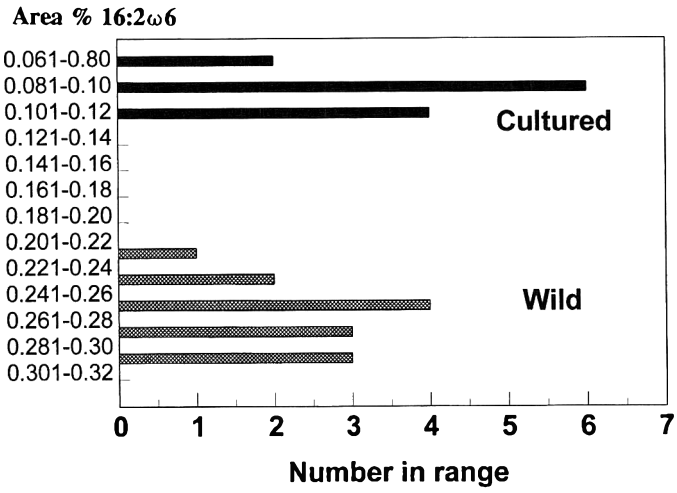

Fig. 1—Distribution of 16:2ω6 in wild and cultured Gulf of Mexico sturgeon.

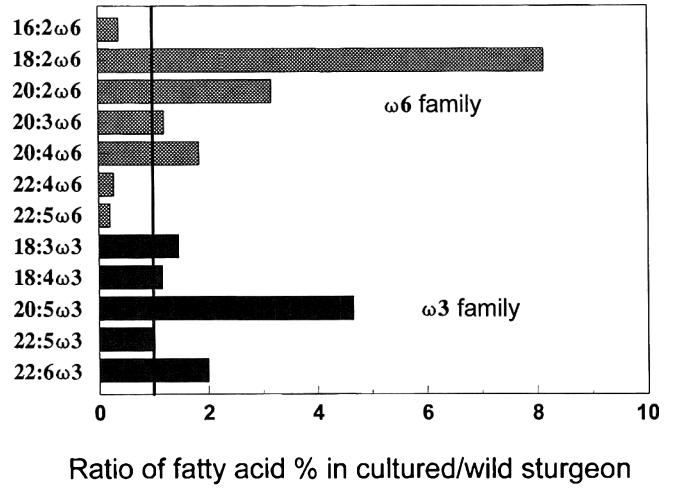

Fig. 2—Relative levels of some ω3 and ω6 fatty acids in cultured and wild Gulf of Mexico sturgeon.

Table 2—Discriminator selection for cultured and wild Gulf sturgeon classification

Variable	Partial R^2	F Statistic	Prob > F	ASCC[a]	Prob > ASCC
16:2ω6	0.9443	390.010	0.0001	0.9443	0.0001
22:5ω6	0.6199	35.886	0.0001	0.9788	0.0001
Phytanic	0.4682	18.492	0.0003	0.9887	0.0001
Tetraene	0.5400	23.475	0.0001	0.9948	0.0001
18:1ω5	0.3292	9.322	0.0065	0.9965	0.0001
17:1ω8	0.3346	9.050	0.0075	0.9977	0.0001
Triene	0.4547	14.177	0.0015	0.9987	0.0001
18:1ω7	0.1284	2.358	0.1442	0.9990	0.0001
16:1ω5	0.2607	5.289	0.0362	0.9992	0.0001
22:1ω9	0.2944	5.842	0.0299	0.9994	0.0001

[a] Average squared canonical correlation

Table 3—Constants for linear discrimination model

Parameter	Cultured mean	Wild mean
Constant ($\hat{\mu}_j$)	−155.70687	−253.58714
16:2ω6 ($\hat{\beta}_{1j}$)	169.41490	1348.000
Phytanic ($\hat{\beta}_{2j}$)	112.07803	5.26948
22:5ω6 ($\hat{\beta}_{3j}$)	−60.77868	116.20500
22:1ω9 ($\hat{\beta}_{4j}$)	85.06918	−79.86866
22:6ω3 ($\hat{\beta}_{5j}$)	11.37510	1.79420

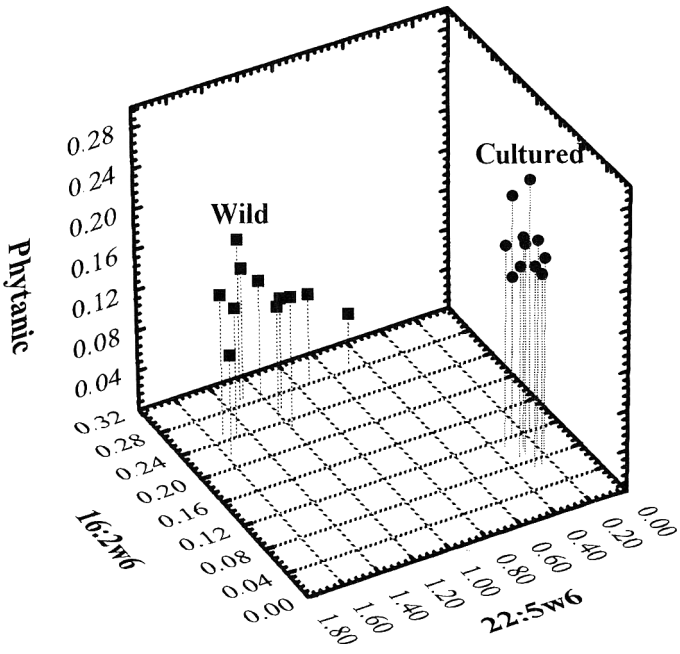

Fig. 3—Discrimination of wild and cultured Gulf sturgeon using 16:2ω6, 22:5ω6 and phytanic acid.

higher levels. Fish can produce these highly unsaturated fatty acids from 18:2w6. Thus we did not expect that their levels would be relatively so low in cultured fish, given the high level of precursor fatty acid present (18:2ω6) in their diets. However, Ackman and Takeuchi (1986) reported similar results for wild and cultured Atlantic salmon smolt. They suggested that the high level of long-chain ω3 fatty acids in cultured fish inhibited elongation and desaturation of 18:2ω6. Both 20:5ω3 and 22:6ω3 were much higher (two to five times) in cultured than wild sturgeon, suggesting that such mechanism could be important in these fish as well. The diet of cultured fish had high levels of long-chain ω3 fatty acids, and most likely they were reflected in high levels in cultured fish flesh. The 16:2ω6 is derived either from chain shortening of 18:2ω6 or through the diet. The level of this fatty acid in cultured fish was almost exactly the same as their diet, suggesting that its origin was dietary. Diets of Gulf sturgeon in the wild have not been analyzed for fatty acid profiles.

The wild fish were caught during their sea-to-river migration, and not the reverse. Gulf sturgeon may spend up to 9 mo in the fresh water Suwannee River. This is highly unusual, since most anadromous fish return to sea shortly after spawning. Analysis of stomach contents of fish caught in the Suwannee River several months after spring migration suggested that they were not feeding and consequently lost weight (Carr et al., 1995). This may be due to unavailability of food in the river and to high water temperature modifying feeding behavior. Thus, fatty acid compositions of fish returning to sea may be quite different from those we analyzed.

Adult sized cultured Gulf sturgeon were not available. Possibly changes in fatty acid compositions during fish growth would affect the discriminator selection and constants for discrimination equations. In addition, information is needed on effects of commercially available diets on fatty acid compositions to develop models that would be applicable to other cultured sturgeon.

CONCLUSION

CULTURED AND WILD Gulf of Mexico sturgeon could be differentiated based on fatty acid compositions. Stepwise discriminant analysis (SDA) of the fatty acid compositional data was useful in determining which fatty acids may identify cultured and wild

populations. However, the general applicability of our SDA model to Gulf of Mexico sturgeon is probably limited due to the small size of the fish sample base. However, a very clear differentiation occurred between wild and cultured fish. Work is ongoing to test effects of diet and fish age on the SDA model.

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Sodium Lactate Affects Shelf Life And Consumer Acceptance Of Fresh Catfish (*Ictalurus nebulosus, marmoratus*) Fillets Under Simulated Retail Conditions

S.K. WILLIAMS, G.E. RODRICK and R.L. WEST

ABSTRACT

Fresh *Ictalurus nebulosus, marmoratus* (Speckled Bullhead catfish) fillets were tumbled under vacuum (172.32 kPa for 15 min) with either 0, 1, or 2% sodium lactate solutions (w/w) adjusted to pH 5.50, and stored at 1.11 ± 1°C for 8 days. Shelf life of fillets treated with 2% sodium lactate was extended from 4 to 7 days. Aerobic plate counts and TBA values were lower ($P < 0.05$) for fillets treated with 2% sodium lactate, compared to controls. Total and fecal coliforms, psychrotrophs, pH, water activity, proximate composition and fatty acid profiles were not affected by sodium lactate. Cooking yields and sodium content were higher for fillets treated with sodium lactate compared to controls.

Key Words: Sodium lactate, catfish, shelf life, bacteria

INTRODUCTION

SODIUM LACTATE extends shelf life by an undefined mechanism (Papadopoulos et al., 1991). Grau (1980) demonstrated that sodium lactate, at pH ≤5.7, inhibited anaerobic growth of *Brochothrix thermosphacta* in beef muscle extract samples at 25° and 5°C. In addition to antimicrobial properties, sodium lactate reportedly depresses water activity (Debevere, 1989). Sodium lactate was effective for growth suppression of *Listeria monocytogenes* (Shelef and Yang, 1991) and *Pseudomonas fragi* (Harmayani et al., 1991). The effectiveness of sodium lactate in shelf life extension of beef products (Papadopoulos et al. 1991; Pagach et al. 1992) and pork sausage formulations (Lamkey et al., 1991; Brewer et al. 1992) has been reported. No research has been reported on effects of sodium lactate on seafood systems. Our objective was to evaluate effectiveness of sodium lactate for maintaining stability of fresh catfish fillets during storage under simulated retail conditions.

MATERIALS & METHODS

Sample preparation

Fresh *Ictalurus nebulosus, marmoratus* (speckled bullhead catfish) fillets were purchased ≈24 hr postharvest. The catfish were harvested from Lake George (Leesburg, Fl.). They were headed, skinned and eviscerated by the harvester. Carcasses were processed into ≈227g fillets at a local seafood market. After receipt of the fillets at the Univ. of Florida laboratory, ≈11-kg aliquots were rinsed in tap water (ambient temperature ≈23°C), placed in the chamber of a 18.2 kg capacity Lyco® vacuum tumbler (model no. 40, Lyco Inc., Columbus, WI) treated with either 0, 1 or 2% sodium lactate solutions adjusted to pH 5.50 with 1M hydrochloric acid, and tumbled under vacuum for 15 min at ≈ 172.32 kPa and 1.67°C. A 60% pure commercial sodium lactate solution (PURAC America Inc., Arlington, IL.) was used to prepare treatments. Sodium lactate solution concentrations were based on total weights of the fillets. Treated fillets were stored in a commercial retail display case (1.11 ± 1°C, model no. S39V1-8U, Kyser Corporation, Conyers, GA) containing crushed ice, and equipped with fluorescent lighting. The temperature of fillets was measured by inserting a thermometer into the thickest muscle area. In an effort to control microbial contamination, the ice was covered with a layer of aluminum foil to prevent direct contact with the fish. Fillets were sprayed each day with a light mist of tap water to control surface dehydration during storage.

Fillets remained in the retail case for ≈10 hr each day. After that, they were removed, placed on ice which had been covered with aluminum foil in 37.8L covered storage containers, and stored at 1.67°C overnight. Three fillets were randomly selected from each treatment group for sampling after 0, 2, 4, 6 and 8 days storage.

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Microbiological analyses

Fillets were analyzed for total aerobic and psychrotrophic organisms; Most Probable Number (MPN) total and fecal coliforms and *Escherichia coli*. Analyses for total aerobic and psychrotrophic organisms were conducted using pre-poured spread plates containing plate count agar (PCA, Difco Laboratories, Detroit, MI). Each fillet was placed in a sterile plastic bag with 100 mL of sterile 0.1% peptone (Difco, Laboratories, Detroit, MI) diluent, and massaged for 1 minute by hand. Serial dilutions were prepared and transferred to pre-poured PCA spread plates for total aerobic and psychrotrophic counts, and to lauryl sulfate tryptose broth MPN tubes for coliform analyses. Plates were incubated at 37°C for 48 hr for aerobic plate count and 20°C for 5 days for psychrotrophic organisms. Following incubation, colonies were counted and data reported as Log colony forming units (CFU)/mL. Total and fecal coliforms and *E. coli* analyses were conducted as outlined in the *Bacteriological Analytical Manual* (U.S. Food & Drug Administration, 1984).

pH

Samples (11g each) were combined with 99 mL deionized water, and ground in a Waring Blendor for 1 min. The sample homogenate was measured for pH using an Orion pH meter (model no. SA520, Fisher Scientific, Pittsburgh, PA). In addition, surface pH was recorded for each fillet with a Fisher Flat Surface Silver/Silver Chloride electrode (Catalog no. 13-620-289, Fisher Scientific, Pittsburgh, PA). All analyses were performed in triplicates.

Sodium analysis

Sodium analysis of fillets was conducted by a modified version of the Stanbio Sodium Procedure (No. 0140, Stanbio Laboratory, Inc, San Antonio, TX) for quantitative and colorimetric determination. Ground fillets (10g) were ashed at 550°C for ≈15 hr. The ash was dissolved in 6M nitric acid. Aliquots (0.5 mL each) of this solution were combined with 2.5 mL of sodium color agent (cat. no. 0141, Stanbio), mixed using a vortex, incubated in an ice water bath for 10 min, centrifuged for 5 min, and equilibrated at room temperature. The appropriate Stanbio reagents were added to the samples and blanks in cuvets (1 cm path) and read at 420 nm within 30 min using a D-40 Beckman Spectrophotometer.

Sensory evaluation and cooking yield

Fillets were rinsed, lightly coated with margarine, and broiled for ≈20 min to internal temperature 74°C (monitored with pre-inserted thermocouples) in a preheated conventional electric oven. Fillets were weighed before and after cooking to determine cooking yield percentages. Samples were sectioned from fillets, and scored for color, texture, flavor, and odor by a 15 to 20 member untrained sensory panel. A 9-point scoring scale was employed, where a score of 9 = excellent, 8 = very good, 7 = good, 6 = below good-above fair, 5 = fair, 4 = below fair-above poor, 3 = poor, 2 = very poor, 1 = extremely poor, and a score of 5 or above = acceptable product.

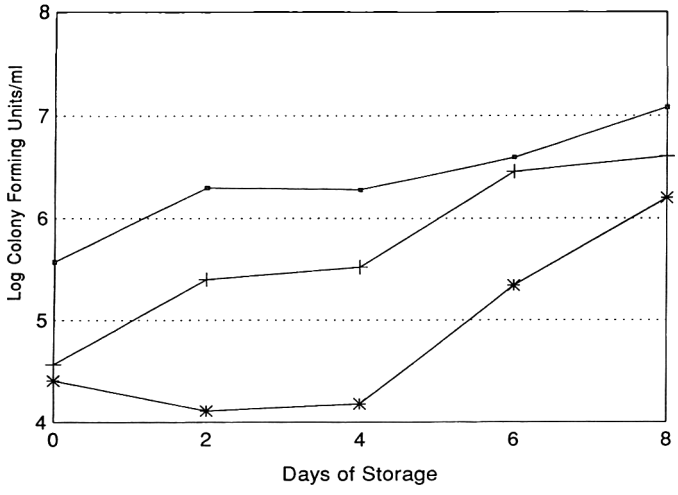

Fig. 1—Aerobic plate counts for fresh *Ictalurus nebulosus, marmoratus* fillets treated with sodium lactate and stored at 1.11 ± 1°C for 8 days. (□ 0% NaL; + 1% NaL; * 2% NaL)

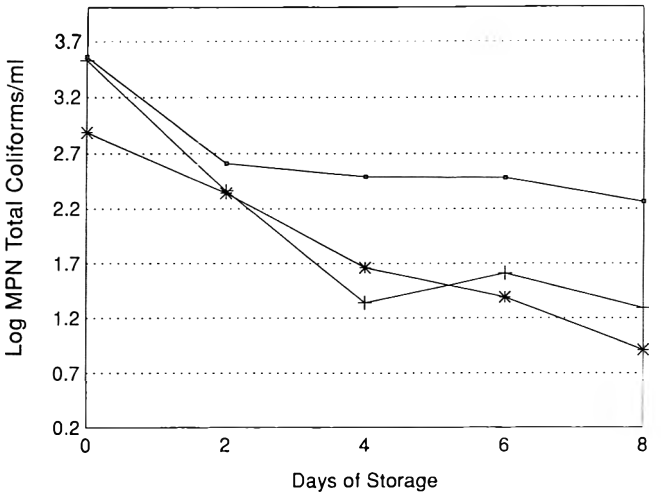

Fig. 3—MPN total coliforms for fresh *Ictalurus nebulosus, marmoratus* fillets treated with sodium lactate and stored at 1.11 ± 1°C for 8 days. (□ 0% NaL; + 1% NaL; * 2% NaL)

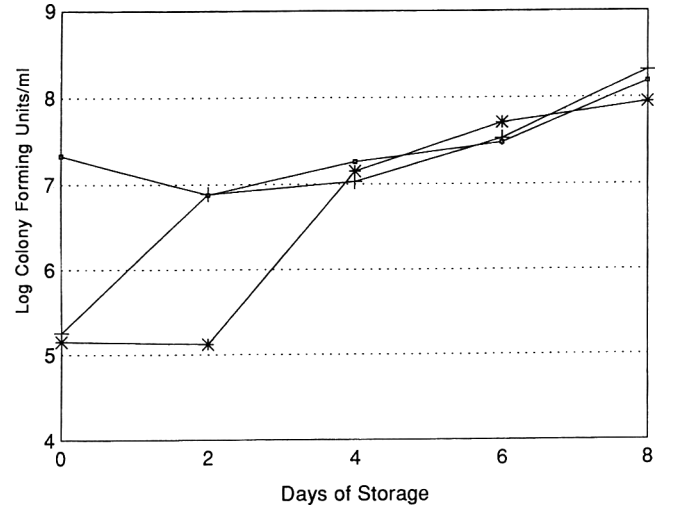

Fig. 2—Psychrotrophic counts for fresh *Ictalurus nebulosus, marmoratus* fillets treated with sodium lactate and stored at 1.11 ± 1°C for 8 days. (□ 0% NaL; + 1% NaL; * 2% NaL)

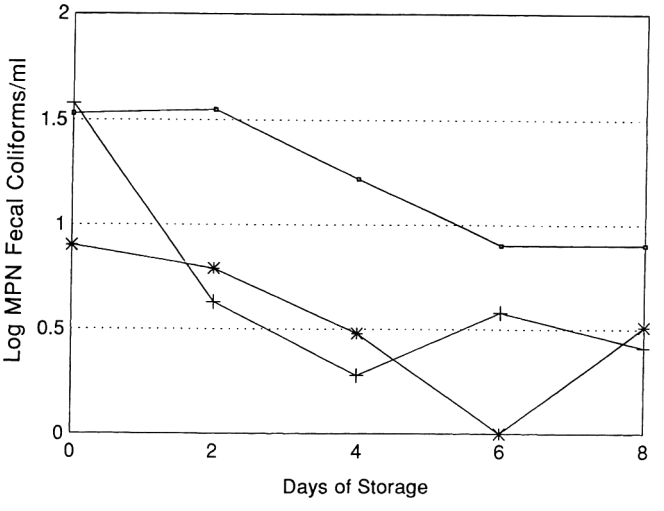

Fig. 4—MPN fecal coliforms for fresh *Ictalurus nebulosus, marmoratus* fillets treated with sodium lactate and stored at 1.11 ± 1°C for 8 days. (□ 0% NaL; + 1% NaL; * 2% NaL)

Water activity

Water activity was measured using a Decagon water activity meter (model CX-1, Decagon Devices, Inc., Pullman, WA). Two 3 cm × 2 cm × 0.5 cm cubes of each fillet were placed in the chamber and measured for water activity. Three fillets were measured/treatment.

2-Thiobarbituric acid analysis

The TBA values (malonaldehyde content) were determined using the 2-Thiobarbituric acid test (Tarladgis et al., 1960) as modified by Rhee (1978). Propyl gallate (PG) and ethylenediaminetetraacetic acid (EDTA) were added during blending to minimize lipid oxidation. Triplicate 10-g samples for each treatment were analyzed. Absorbance was read at 530 nanometers using a D-40 Beckman Spectrophotometer. Results were expressed as mg malonaldehyde/kg sample.

Chemical analyses

Analyses for moisture (No. 24.003), protein (No. 24.027), fat (No. 18.044), ash (No. 18.025) and fatty acid profiles (No. 18.059) were performed after 0 days storage in the retail display case. Analyses were conducted as outlined in the *Official Methods of Analysis of the AOAC* (AOAC, 1984). Three fillets were ground/treatment. Each ground sample was analyzed in duplicate. No statistical analyses were conducted on chemical analytical data.

Data analysis

The data were analyzed using the 6.04 edition of the Statistical Analysis System (SAS Institute, Inc., 1990). Statistical methods included Analysis of variance and Duncans Multiple Range Test (Littell et al., 1991). A 3 × 5 factorial design with three replications was employed. The levels of sodium lactate (0, 1 and 2%) and storage time (0, 2, 4, 6, 8, or 7 days for sensory evaluation) were main effects. The interactions between treatments and storage time were tested.

RESULTS & DISCUSSION

Microbial growth

The APCs for fillets treated with 2.0% sodium lactate were lower ($p < 0.05$) than control fillets through 8 days storage (Fig. 1). The APCs were similar ($P > 0.05$) for fillets treated with 1 and 2% sodium lactate. Except for days 0 and 2, sodium lactate had no antimicrobial effect ($P > 0.05$) on psychrotrophic organisms (Fig. 2) Initially (i.e. day 0), all sodium lactate treatments resulted in lower ($P < 0.05$) psychrotrophic organisms, when compared to controls. After 2 days storage psychrotrophic counts for fillets treated with 2% sodium lactate remained lower ($P < 0.05$) than controls and fillets treated with 1% sodium lactate. After 4 days storage psychrotrophic counts were similar ($P > 0.05$) for all treatments.

Table 1—Mean sodium values for fresh *Ictalurus nebulosus marmoratus* fillets treated with sodium lactate (NaL) and stored at 1.11 ± 1°C for 8 days

Processing date	Treatment % NaL	Sodium (mg/100g)[a]
Nov., 1991		
	0	98.68a
	1	241.23b
	2	338.22c
Feb., 1992		
	0	110.41a
	1	226.69b
	2	310.47c
Mar., 1992		
	0	100.43a
	1	208.73b
	2	308.53c
Overall Means:		
	0	103.17a
	1	227.55b
	2	319.09c

[a] Means in the same column per processing date bearing the same letter are not significantly different (P>0.05).

Table 2—Mean panelists responses for fresh *Ictalurus nebulosus, marmoratus* fillets treated with sodium lactate and stored at 1.11 ± 1°C for 7 days

		Panelists' responses Percent sodium lactate[b]		
Attributes[a]	Day	0	1	2
Color	0	7.36	7.48	7.21
	2	7.22	7.86	7.58
	4	6.36	7.34	7.64
	6	—	7.04	6.79
	7	—	6.32	6.71
Texture	0	7.11	7.03	7.26
	2	7.50	7.46	7.49
	4	7.58	7.61	7.64
	6	—	6.84	7.06
	7	—	6.01	6.17
Flavor	0	6.78	6.78	6.84
	2	6.78	7.48	7.38
	4	6.18	6.57	7.10
	6	—	6.54	6.64
	7	—	5.09b	6.17a
Odor	0	6.85	6.81	6.83
	2	7.03	7.50	7.73
	4	6.13	6.84	7.17
	6	—	6.24	6.86
	7	—	4.71b	6.46a

[a] Means on the same horizontal line bearing the same letter are not significantly different (P>0.05).

[b] (—) no test was conducted. See Material & Methods for complete Scoring Scale.

The antimicrobial effects of sodium lactate were limited for total and fecal coliforms (Fig. 3 and 4). Total coliform counts for fillets treated with 2% sodium lactate were lower ($P < 0.05$) than controls and fillets treated with 1% sodium lactate after 8 days storage (Fig. 3). Fecal coliforms were similar ($P > 0.05$) for all treatments, and remained at <2 log counts through 8 days storage (Fig. 4).

Sodium analyses

The addition of sodium lactate resulted in notable increases in total sodium (Table 1). Fillets treated with 1 and 2% sodium lactate resulted in average sodium increases of 124.38 mg/100g (120% increase) and 215.92 mg/100g (209% increase), respectively, when compared to untreated controls. The 103.17 mg/100g mean sodium content of controls was similar to the mean sodium value of 98 mg/100g reported by Mustafa and Medeiros (1985) for Channel catfish (*Ictalurus punctatus rafinesque*). Sodium values of 63 and 100 mg/100g were reported in the USDA Handbook no. 8 (Watt and Merrill, 1987) for freshwater and ocean channel catfish meat, respectively.

Sensory evaluation

No color, texture, flavor or odor differences ($P > 0.05$) were revealed between controls and fillets treated with 1 and 2% sodium lactate through 4 days storage (Table 2). Development of an intense off-odor in controls after 5 days resulted in cancellation of sensory evaluation of controls. Fillets treated with 1 and 2% sodium lactate were rated acceptable in color, texture, flavor and odor through 7 days storage. After 7 days, flavor and odor of fillets containing 1% sodium lactate had declined ($P < 0.05$) compared to fillets treated with 2% sodium lactate. Some of the panelists (25%) detected a 'fishy' and/or 'muddy' aftertaste in fillets treated with 1% sodium lactate. These flavor and odor differences were not reported for fillets treated with 2% sodium lactate.

It was noted by ≈10% of panelists throughout the study that fillets treated with 2% sodium lactate had an objectionable aftertaste, which was described as 'metallic', 'sodium' and/or 'chemical'. This aftertaste was not reported in controls and fillets treated with 1% sodium lactate. Almost 60% of the panelists preferred the flavor imparted by the sodium lactate. They reported that fillets with the sodium lactate had more flavor.

Table 3—Mean cooking yield percentages for fresh *Ictalurus nebulosus, marmoratus* fillets treated with sodium lactate and stored at 1.11 ± 1°C for 8 days

Storage day[a]	Sodium lactate (%)[b] 0	1	2
0	76.20	75.49	77.62
2	77.01	75.98	78.57
4	76.06	75.21	77.39
6	—	73.56	76.60
7	—	73.48	75.80

[a] Means on the same horizontal line bearing the same letter are not significantly different (P>0.05).

[b] (—) no test was conducted.

Table 4—Mean 2-Thiobarbituric Acid values for fresh *Ictalurus nebulosus, marmoratus* fillets treated with sodium lactate and stored at 1.11 ± 1°C for 8 days

Storage Day[a]	TBA values (mg malonaldehyde/kg) Sodium Lactate (%) 0	1	2
0	0.30	0.30	0.23
2	0.48a	0.51a	0.35b
4	0.59a	0.59a	0.53b
6	0.82a	0.69b	0.64b
8	1.17a	1.26b	0.96c

[a] Means on the same horizontal line bearing the same letter are not significantly different (P>0.05).

Table 5—Mean percents for proximate composition of fresh *Ictalurus nebulosus, marmoratus* fillets treated with sodium lactate and stored at 1.11 ± 1°C for 8 days

Processing date	Treatment % NaL[a]	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Nov., 1991					
	0	83.99	13.40	1.70	0.91
	1	83.44	13.90	1.65	1.01
	2	81.97	14.70	1.50	1.89
Feb., 1992					
	0	83.43	13.50	2.10	0.97
	1	83.48	13.20	2.18	1.14
	2	81.65	14.30	2.20	1.85
Mar., 1992					
	0	82.33	14.90	1.90	0.87
	1	83.45	13.60	1.86	1.09
	2	82.18	14.10	1.80	1.92
Overall means:					
	0	83.25	13.93	1.90	0.92
	1	83.45	13.57	1.90	1.08
	2	81.93	14.37	1.83	1.89

[a] NaL = sodium lactate.

Cooking yield

No significant differences ($P > 0.05$) in cooking yields were revealed between controls and fillets treated with 1 and 2% so-

Table 6—Mean fatty acid profiles for fresh *Ictalurus nebulosus, marmoratus* fillets treated with sodium lactate and stored at 1.11 ± 1°C for 8 days

Fatty acid	Percent fatty acid in fat Sodium Lactate (%) 0	2
Saturated, total	38.10	32.20
6:0 Caproic acid	0.00	0.00
8:0 Caprylic acid	0.00	0.00
10:0 Capric acid	0.00	0.00
12:0 Lauric acid	0.40	0.20
14:0 Myristic acid	4.00	3.10
16:0 Palmitic acid	27.90	23.70
18:0 Stearic acid	3.80	3.70
20:0 Arachidic acid	0.50	0.40
22:0 Behenic acid	0.00	0.00
24:0 Lignoceric acid	1.50	1.10
Monounsaturated, total	38.80	44.00
14:1 Myristoleic acid	0.00	0.20
16:1 Palmitoleic acid	13.20	10.60
18:1 Oleic acid	24.70	32.40
20:1 Eicosenoic acid	0.90	0.80
22:1 Euric acid	0.00	0.00
Polyunsaturated, total	20.50	21.70
18:2 Linoleic acid	4.70	5.90
18:3 Linolenic acid	9.70	11.30
20:4 Arachidonic acid	2.90	2.30
20:5ω3 Eicosapentaenoic acid	0.00	0.00
22:6ω3 Docosahexaenoic acid	3.20	2.20
Total fatty acids	97.40	97.90

dium lactate through 4 days storage (Table 3). A trend was observed, that cooking yields for fillets treated with 2% sodium lactate were higher through 4 days storage, than controls and through 8 days storage, compared to fillets treated with 1% sodium lactate.

Chemical analyses

The data demonstrated no effects ($P > 0.05$) of sodium lactate on water activity. Mean water activity values over 8 days storage were 0.990, 0.989, and 0.992 for controls, and fillets treated with 1 and 2% sodium lactate, respectively. Similar trends were determined for pH. No differences ($P > 0.05$) were revealed between surface pH and corresponding total pH for any treatments through 8 days storage. The initial pH values were 6.50, 6.30, and 6.32 for the control fillets and those treated with 1 and 2% sodium lactate, respectively.

TBA values for fillets increased with storage time (Table 4). The data demonstrated lower ($P < 0.05$) TBA values for fillets treated with 2% sodium lactate after 2 days, and through 8 days storage, compared to fillets treated with 0 and 1% sodium lactate.

Except for protein, proximate values were similar to those reported by Kinsella et al. (1977) for Brown Bullhead catfish (*Ictalurus nebulosus*) (Table 5). Sodium lactate treatments resulted in increased ash.

The fatty acid profile differed slightly for untreated and treated fillets (Table 6). Those treated with 2% sodium lactate had slightly higher fatty acid components. The total saturated fatty acids were 5.90% higher for controls when compared to those treated with 2.0% sodium lactate; and the monounsaturated fatty acids were 5.20% higher for fillets treated with 2.00% sodium lactate. Total polyunsaturated fatty acids of fillets treated with 2.00% sodium lactate were 1.20% higher than controls.

CONCLUSION

THE SHELF LIFE of catfish fillets treated with 2.00% sodium lactate (i.e. 7-day shelf life) was extended 3 days, compared to controls. The 2.00% sodium lactate treatment resulted in initial lags in total APCs (4 days) and psychrotrophic organisms (2 days). The sodium lactate treatment also retarded rancidity, but had no effect on water activity during 8 days storage. Except for ash, the proximate composition and fatty acid profiles were not affected by sodium lactate. A notable increase in sodium was attributed to the sodium lactate. Results demonstrated that sodium lactate may be successfully employed in fresh *Ictalurus nebulosus, marmoratus* fillets as an antimicrobial agent and an antioxidant.

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Viscoelastic Property Changes in Cheddar Cheese During Ripening

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ABSTRACT

The rheological properties of pasteurized and raw milk Cheddar cheese were studied using oscillatory dynamic measurements, and a specially designed rheometer fixture that prevented specimen slippage. Dynamic measurements within the linear viscoelastic range were made throughout ripening. Within-cheese changes, as related to ripening time, as well as between-cheese-type differences in G' and G" were observed. Differences in rheological characteristics were attributed to proteolytic activities in Cheddar cheese during ripening. Specific peptide profiles associated with proteolysis during ripening may affect cheese rheological properties.

Key Words: Cheddar cheese, rheology, viscoelasticity, proteolysis, oscillatory measurements

INTRODUCTION

TEXTURAL CHARACTERISTICS affect cheese eating quality, usage, and handling (Walstra and Peleg, 1991; Visser, 1991). Rheological properties of cheese are affected by the composition and structural characteristics and undergo notable changes during ripening. These variations reflect effects of chemical and physical changes during ripening and involve all major cheese components (Christensen et al., 1991). In Cheddar cheese, proteolysis has been shown most important in that respect (Fox, 1989).

Determining rheological properties of cheese is complicated by structural heterogeneity (Walstra and van Vliet, 1982) Cheese is viscoelastic and in most cases shows no notable yield stress. The viscoelastic nature of cheese implies that the ratio of elastic to viscous characteristics is related to the time scale of deformation (Walstra and van Vliet, 1982; Walstra and Peleg, 1991). Cheese and Cheddar cheese rheology have been mainly studied using uniaxial compression (Chen et al., 1979; Imoto et al., 1979; Green et al., 1981; Amantea et al., 1986; Lin et al., 1987; Bertola et al., 1991), and the concept of texture profile analysis has been widely used (Bourne, 1982). Factors which affected cheese texture included composition, cheesemaking condition, and extent of proteolysis during ripening.

Testing materials within their linear viscoelastic range, and assuming even strain distribution, measurements enable better understanding of relationships between structural and rheological characteristics. Within the linear range, viscoelastic characteristics are independent of magnitude and rate of strain. This presents an advantage over traditional compression type measurements (Ferry, 1980; Green et al., 1989). Advanced instrumentation enables viscoelastic properties of food systems to be measured using dynamic rheological tests to measure storage moduli (G') and loss moduli (G") (Ferry, 1980; Rao and Steffe, 1992).

Taneya et al. (1979) reported on viscoelastic properties of Cheddar and Gouda cheeses. Masi and Addeo (1986) reported on such properties of Italian cheeses, and Visser (1991) on characteristics of Gouda cheese. Dynamic rheological measurements were used by Tunick et al. (1990) to study textural characteristics of lowfat Mozzarella. Problems related to specimen slippage during dynamic rheological analysis were considered by Nolan et al. (1989).

Our objective was to develop a methodology to determine rheological properties of Cheddar cheese using a controlled-stress rheometer, and to compare rheological characteristics of cheeses prepared from pasteurized or raw milk as related to ripening time.

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MATERIALS & METHODS

CHEDDAR CHEESES prepared from pasteurized milk (PCH) and raw milk (RCH) were obtained from Vella Cheese Company (Sonoma, CA). Both types were manufactured using the same procedures, and for each, cheese from the same cheese vat was used. Cheese ripening in the plant was carried out at 5°C under vacuum in plastic bags. PCH at 7 days and RCH at 95 days were delivered to our laboratory at temperature 6–7°C. These were immediately cut into 3-kg blocks, packed under vacuum in BK1 cheese bags (Cryovac Division, W.R. Grace Co., Hayward, CA) and stored for ripening at 5°C. PCH were studied at cheese ages 19, 240, and 470 days and were denoted PC, (control) PM, (mild) and PS, (sharp) respectively. Likewise RCH was studied at 246 and 475 days and samples were denoted RM and RS, respectively.

Analyses

Cheese composition (moisture, fat, ash, salt, and protein) was determined using Standard Methods (Bradley et al., 1992, Table 1). All analyses were carried out in quadruplicate.

Proteolysis during cheese ripening was monitored using cheese fractionation methods reviewed by Christensen et al. (1991). Sodium citrate-soluble N-fraction of cheese was prepared using a modification of the method described by Vakaleris and Price (1959) and later by Nooman (1977). Fifty g cheese, 200 mL of sodium citrate solution in distilled water (0.5M, pH 8.75–8.8) and 400 mL distilled water were homogenized using a Waring Blendor (Dynamics Corp., New Hartford, CT) for 3 min at maximum speed. The homogenate was stirred 30 min at 25°C. A 400-mL portion was adjusted to pH 4.6 by adding 1.41N HCl and the volume was adjusted to 500 mL in a volumetric flask. The mixture was centrifuged 20 min at 3000 × *g* and the supernatant filtered through Whatman No. 1 paper (Fisher Scientific, Pittsburgh, PA). The filtrate was denoted as pH 4.6 N-soluble fraction. The N content in the filtrate was determined by the Kjeldahl method (Bradley et al., 1992) and expressed as % of cheese total N determined by the same method.

The pH 4.6-soluble N-fraction was used for further fractionation. Trichloroacetic acid-soluble N-fractions (2% and 12%) were prepared using methods described by Kuchroo and Fox (1982a,b). The N content was determined as described and expressed as % of cheese total N content. These fractions were denoted 2% TCA and 12% TCA.

The cheese N fraction soluble in 70% ethanol was prepared from the pH 4.6-soluble N-fraction. 93.4 mL 100% ethanol was added to 40 mL of pH 4.6 citrate-soluble N-fraction. After incubation for 12 hr at 25°C the mixture was centrifuged (3000 × *g*) and the supernatant treated as described. The filtrate was denoted as 70% ethanol-soluble N-fraction.

The 5% PTA-soluble N-fraction was prepared using the procedure described by Kuchroo and Fox (1982a,b) with some modifications. Thirty-five mL of 3.95M H_2SO_4 and 15 mL of 33.3% (weight/weight) (PTA) (Sigma Chemicals Co., St. Louis, MO) were added to 50 mL of the pH 4.6 citrate-soluble N-fraction and the mixture was incubated for 12 hr at 25°C. The mixture was centrifuged at 3000 × *g* for 20 min, the

Table 1—Proximate composition of Cheddar cheeses from pasteurized milk or raw milk

Cheese	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	Salt (%)
PC[a]	39.1 ± 0.2	32.5 ± 0.3	22.9 ± 0.09	3.05 ± 0.02	1.28 ± 0.03
PM	39.3 ± 0.1	32.2 ± 0.5	23.1 ± 0.09	3.06 ± 0.05	1.30 ± 0.03
PS	39.3 ± 0.3	32.1 ± 0.4	23.4 ± 0.07	3.23 ± 0.03	1.45 ± 0.02
RM	38.9 ± 0.3	32.0 ± 0.6	23.2 ± 0.08	3.02 ± 0.04	1.34 ± 0.01
RS	39.1 ± 0.4	31.8 ± 0.8	23.5 ± 0.08	3.15 ± 0.02	1.31 ± 0.01

[a] PC, PM, and PS are pasteurized milk cheeses after 19, 240, and 470 days, respectively. RM and RS are raw milk cheeses after 246 and 475 days, respectively.

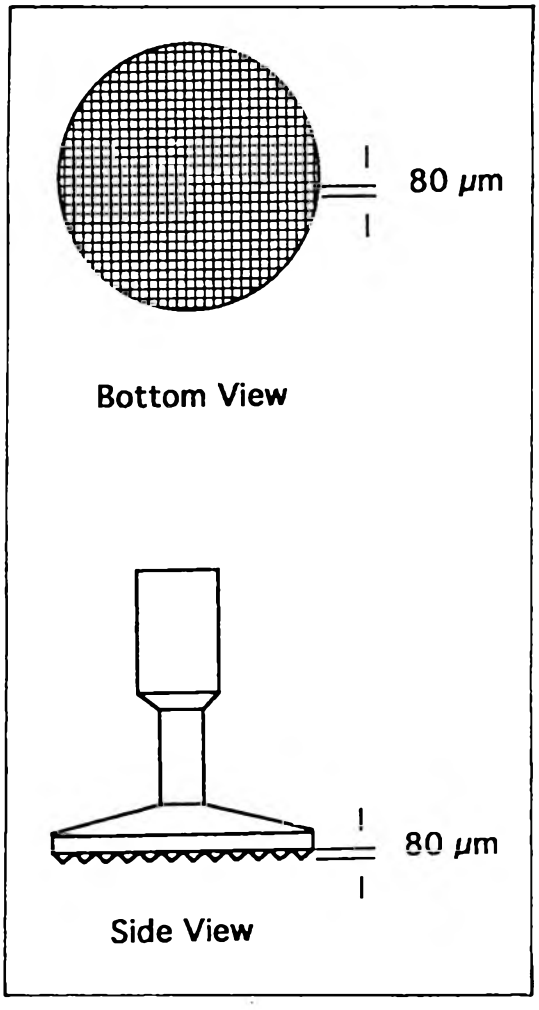

Fig. 1—Upper rheometer plate machined to provide a roughened surface to prevent sample slippage during oscillation tests.

supernatant was filtered through Whatman No. 1 paper. The N content of the filtrate was determined as described and expressed as % of cheese total N. This fraction was denoted 5% PTA. All analyses were carried out in quadruplicate.

Dynamic rheological measurements

The viscoelastic properties of Cheddar cheese were studied using a controlled-stress rheometer (Model CSL100 Carri-Med CSR, TA Instruments, New Castle, DE). A special upper plate (4 cm dia) with a rough surface was manufactured by the Food Science & Technology Dept. mechanical shop (Fig. 1). Cheddar cheese samples (2 cm diam) were prepared (at 5°C) using a cork borer. These sample cylinders were immediately cut to lengths of 2, 5, or 10 mm using a template and a 0.2-mm stainless steel cutting device. Samples were wrapped in aluminum foil and tempered for 5 hr to the test temperature. The temperature of the lower plate of the rheometer was adjusted accordingly. Log frequency sweep and torque sweep were carried out to characterize rheological characteristics of the PC, PM, PS, RM, and RS cheeses. All measurements consisted of 5 duplicates. For log frequency sweep, frequencies ranging from 0.1 to 10 Hz were used, and measurements were carried out at 10°C, 15°C, 25°C, and 35°C at a torque of 700 μn-m.

Table 2—Soluble nitrogen (% of total N) in different cheese extracts

Cheese[f]	pH=4.6	2% TCA	12% TCA	70% ETOH	5% PTA
PC	11.59[e]	9.58[e]	8.56[d]	8.36[d]	4.82[e]
PM	29.14[b]	25.37[c]	22.10[c]	21.61[b]	12.45[c]
PS	36.15[a]	32.85[a]	30.50[a]	29.41[a]	17.22[b]
RM	20.09[d]	23.14[d]	21.63[c]	21.12[c]	11.90[d]
RS	28.76[c]	31.00[b]	29.63[b]	29.35[a]	17.67[a]

[a,b,c,d,e] Means within same column followed by different superscripts are different ($P < 0.05$).

[f] PC, PM, and PS are pasteurized milk cheeses after 19, 240, and 470 days, respectively. RM and RS are raw milk cheeses after 246 and 475 days, respectively.

Torque sweep measurements were carried out at 25°C at a frequency of 25 rad. Temperature sweep results were obtained at a frequency of 25 rad and a torque of 700 μn-m. A custom-made round plastic compartment was installed to prevent drying and to maintain consistent temperature throughout each sample. The rheological parameters G' and G" were determined in all cases.

Statistical analyses

Significance of chemical and rheological test results were analyzed using the ANOVA test procedure included in the SigmaStat® software (Jandel Scientific, San Rafael, CA).

RESULTS & DISCUSSION

Proteolysis during ripening

Cheddar cheese ripening is associated (among other things) with an appreciable extent of proteolysis that involves all of the major caseins to different extents (Fox, 1989). The results of crude fractionation of N-components in the cheese were compared at various stages of ripening (Table 2). An increase in proportion of N-compounds occurred as ripening time increased.

For both PCH and RCH, an increase in the proportion of soluble N-compounds was evident ($P < 0.05$) as ripening time increased, in all of the cheese extracts. This increase indicates the progress of proteolysis during ripening (Fox, 1989). The N-content of the fraction soluble at pH 4.6 represents a heterogeneous mixture of proteolysis products as well as whey proteins. It has been reported to increase with ripening time, and the ratio between soluble N to total N has been suggested as a ripening index (Kuchroo and Fox, 1982a, b; Nooman, 1977; Reville and Fox, 1978). At all comparable ages, the proportion of N-components soluble at pH 4.6 was higher in the PCH than in the RCH ($P < 0.05$).

The proportion of N-containing compounds soluble in 2% or 12% TCA increased in both PCH and RCH as with increased ripening time, and higher proportions of these compounds occurred in PCH than in RCH ($P < 0.05$). The fraction soluble in 2% TCA represents a mixture of medium and small peptides, and that soluble in 12% TCA represents a mixture of small peptides and free amino acids (Christensen et al., 1991; Kuchroo and Fox, 1982b). Results of fractionation with 70% ethanol, representing a mixture of small peptides and amino acids, were very similar to those with 12% TCA as has previously reported (Reville and Fox, 1978). Higher levels of N components soluble in 70% ETOH were found in the PCH than in the RCH ($P < 0.05$).

The proportion of N components soluble in 5% PTA, representing a mixture of peptides (< 600 Dalton) and amino acids (Jarret et al., 1982; Christensen et al., 1991), increased with ripening time. The proportion of these compounds in PCH was

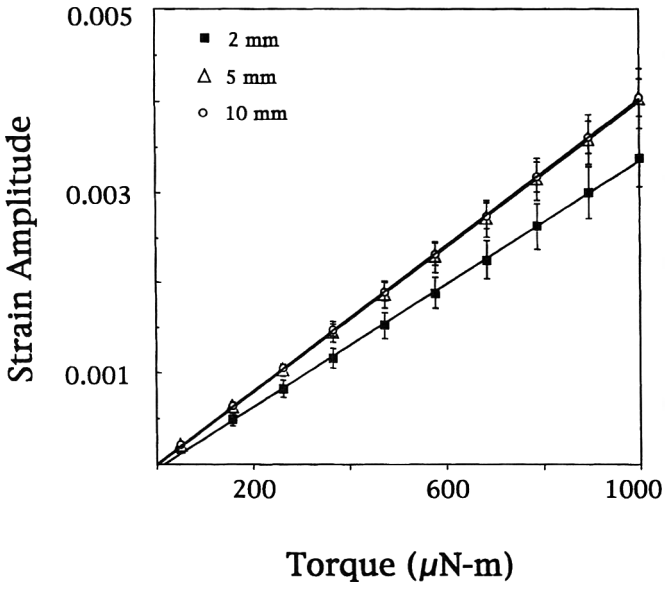

Fig. 2—Strain amplitudes of Cheddar cheese samples as related to sample thickness and torque during oscillatory testing.

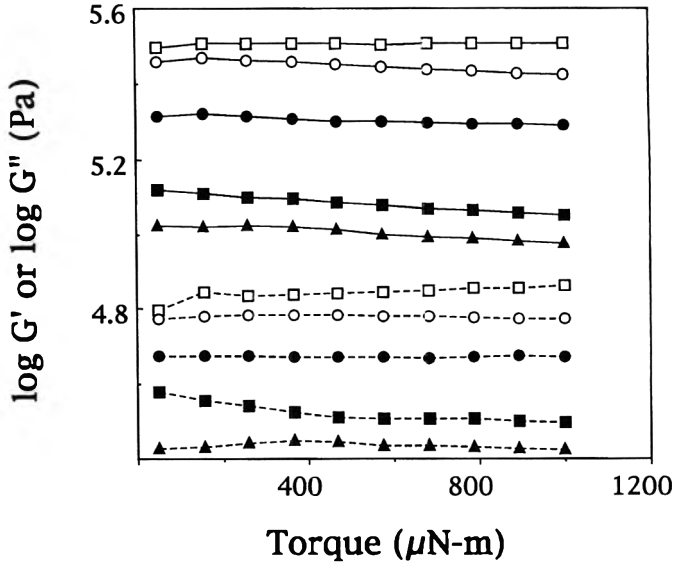

Fig. 3—Linear viscoelastic ranges of different Cheddar cheese samples, made from pasteurized (P) and raw (R) milk and either fresh (C), mild (M), or sharp (S). (See text for ages). Symbols: □ RM; ○ RS; ● PS; ■ PM; ▲ PC; ——— G'; ----- G".

slightly, higher than in RCH after 240 ripening days ($P < 0.05$). After 470 ripening days the level of these compounds in RCH was slightly higher than in PCH ($P < 0.05$).

The results of crude fractionation analyses indicated that, although in both RCH and PCH the extent of proteolysis progressed with ripening time, differences were evident. These may indicate the effects of pasteurization on proteolytic activities in milk. Inactivation of proteinase inhibitors (Nooman, 1975) or activation of plasminogen during pasteurization (Rollema et al., 1981) probably account for the differences.

Dynamic viscoelastic measurements

A critical difficulty in dynamic rheological measurements that had adversely affected results was specimen slippage (Navickis and Bagley, 1983; Nolan et al., 1989; Tunick et al., 1990). Anchoring of cheese specimen to the measuring device has been accomplished by gluing them to rheometer plates (Nolan et al., 1989; Tunick et al., 1990). In our study, a special upper plate was developed (Fig. 1) to ensure proper specimen anchoring. In a preliminary test, the effect of specimen height on measurement was evaluated. Results of a strain sweep measurement using three specimen heights (Fig. 2) indicated that, regardless of height, a linear ($R^2 = 1$) relationship between strain amplitude and applied torque was obtained. However, results using the 2-mm sample height were appreciably different from those when 5-mm or 10-mm specimens were used. Results with 5-mm and 10-mm samples were identical. These results indicated that, below 5 mm, results of dynamic oscillatory tests were affected by specimen dimensions, probably due to "end effects" associated with specimen anchoring. Based on these results, and in order to minimize risk of such effects, a 10-mm thickness was selected. Evidence of specimen slippage was not detected in any case; the special upper plate that we developed provided a suitable solution. It could therefore replace the specimen gluing practice.

Linear viscoelastic range

Neither G' or G" for cheeses of different ages were affected by the magnitude of torque (Fig. 3), thus indicating that the cheeses behaved as linear viscoelastic substances during dynamic testing at torques of 1000 μn-m or less. Differences in both G' and G" were identified among most cheeses. At very low torques (50 μn-m) differences ($P < 0.05$) were found in G' of different cheeses except for pairs PM/PC and RM/RS. At higher torque values, among-cheese differences ($P < 0.05$) in both G' and G" were evident. The highest G' values were obtained for RM followed by RS>PS>PM>PC. The G" values for the different cheeses were in the same among-cheeses order as those of G'. The results of the torque sweep experiments suggested that, regardless of chemical and structural changes during ripening, no deviation from linear viscoelasticity was noted for torques of 1000 μn-m or less.

Viscoelastic properties as related to frequency and temperature

In all cases, G' was affected by temperature, cheese age, and frequency (Fig. 4 and Fig. 5). For both PCH and RCH, a larger elastic response was observed as related to frequency. These results were in agreement with those reported by Taneya et al. (1979). Both G' and G" were inversely proportional to temperature. This could be attributed to the effect of temperature on both fat and proteinaceous components.

Results of the frequency sweep tests indicated that G' of both PCH and RCH varied, related to ripening time. Except in a few cases (PC and PS at 0.63 rad and 10°C; PM and PS at 35°C and 0.63 or 63 rad), for a given temperature, differences ($P < 0.05$) in G' were found among pasteurized cheeses of different ages. For PCH, an increase in G' was observed as related to cheese age and indicated an increase in manifestation of elastic structural elements. The increase in G' as related to cheese age likely represents the effects of accumulation of proteolysis products (Table 2). The increase in expression of elastic components in the cheese with ripening time could probably be attributed to compacting associated with protein degradation. In addition, for each peptide bond cleaved in the course of proteolysis, two ionic groups would be formed, to which water was bound. This would result in a decrease in proportion of water available to act as lubricant and could also explain the increase in G' with ripening (Stanley and Emmons, 1977; Creamer and Olson, 1982). In the case of RCH, higher G' values were obtained with RM than with RS ($P < 0.05$). For both PCH and RCH, G" was almost independent of frequency at 10°C and 15°C. However, it exhibited an increase with frequency at 25°C and 35°C.

The effect of temperature on G' and G" was measured at frequency 25 rad and torque 700 μn-m (Fig. 5). Both storage modulus and loss modulus were inversely proportional to tem-

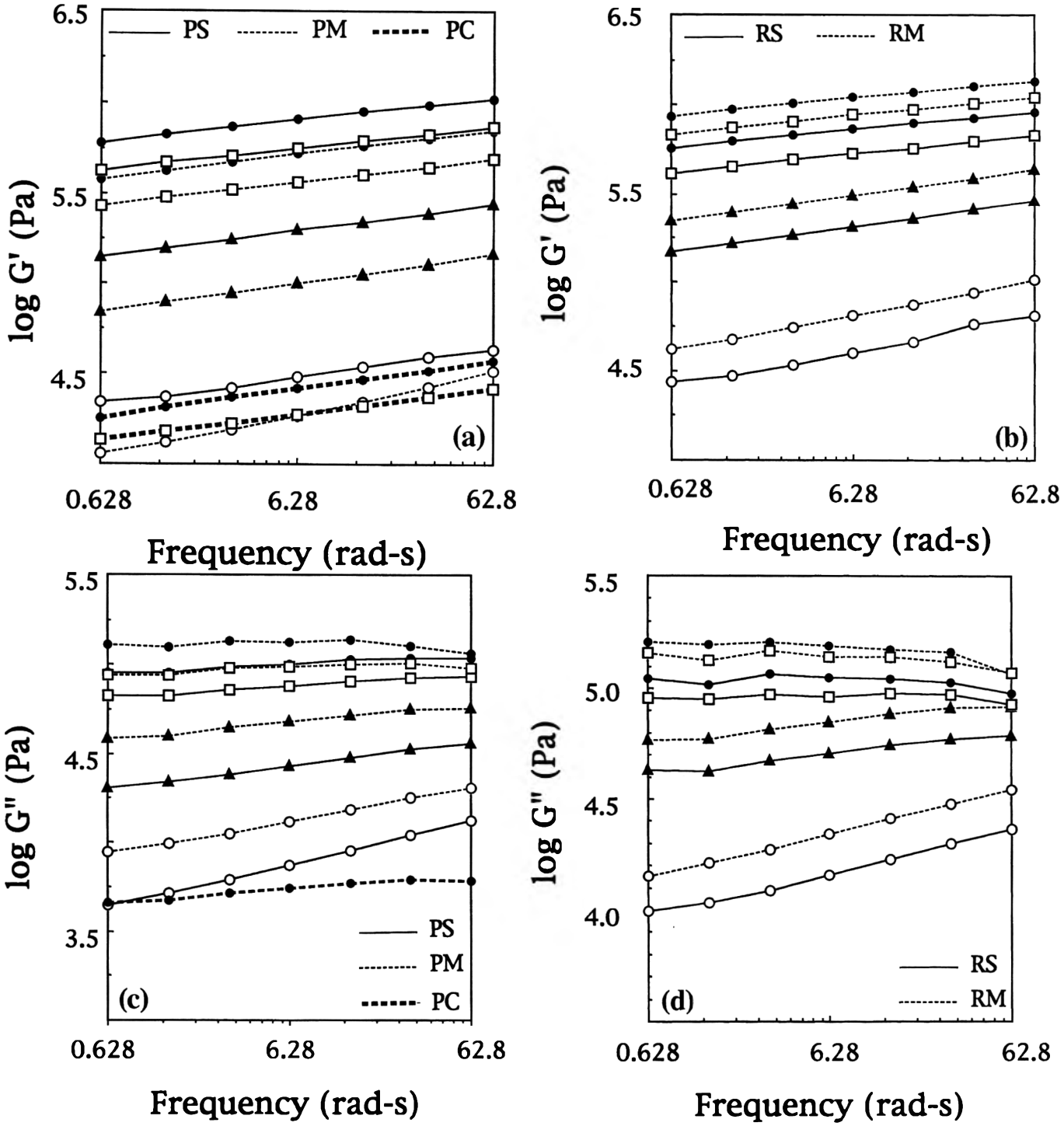

Fig. 4—The viscoelastic properties of Cheddar cheeses as related to frequency of oscillation and temperature: storage moduli of cheeses from pasteurized milk (a) or from raw milk (b) and loss moduli of cheeses from pasteurized milk (c) or from raw milk (d). Symbols: ● 10°C; □ 15°C; ▲ 25°C; ○ 35°C.

perature, thus indicating the softening of the matrix with increasing temperatures. At each temperature, the G' of PM cheese was the lowest while the G' of the RM was the highest ($P < 0.05$). The storage moduli of PS and RS cheeses were similar at 10 to 25°C. A similar among-cheeses pattern was observed for the G" results.

For PCH cheeses, the higher G' values of PS in comparison to PM cheese may be related to the higher proteolysis in PS as evident from results (Table 2). For the raw milk cheeses, differences in G' between RM and RS were not in agreement with results of proteolysis analyses. Although the extent of proteolysis was higher in RS than in RM cheese, the expression of elastic behavior was greater in the RM. The trends observed regarding G" were similar to those observed for G'. Effects of temperature and cheese age on G' we observed for the PCH were similar to these reported by Taneya et al. (1979) on Cheddar cheese. A decrease in both G' and G" with increasing temperature was also reported by Hsieh et al. (1993) on Mozzarella cheese.

In a separate study on the same cheeses the peptide profiles were studied by reverse-phase HPLC (Wang et al., 1993). Results indicated differences in peptide profiles between pasteurized and raw milk cheeses and suggested that different proteolytic activities were involved in ripening. Such differences may explain the different patterns observed between pasteurized and the raw milk cheeses and the effects of ripening time on

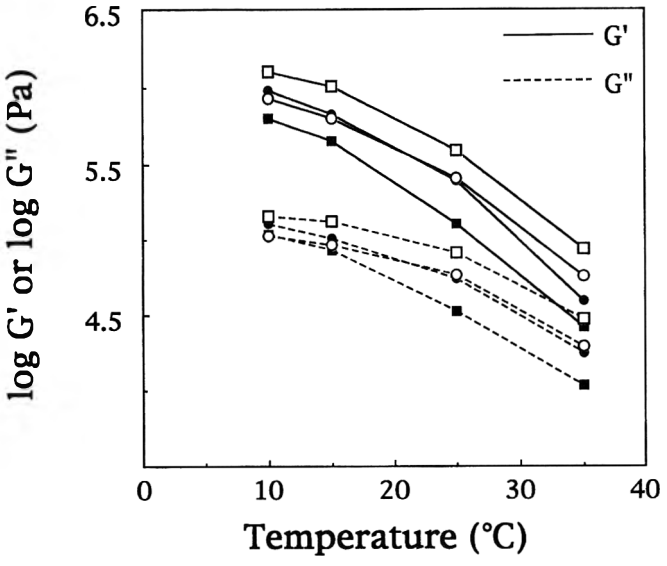

Fig. 5—Effect of temperature on viscolastic properties of mild (M) and sharp (S) Cheddar cheeses from pasteurized (P) and raw (R) milks. Symbols: □ RM; ○ RS; ● PS; ■ PM.

viscoelastic characteristics. Among-cheese differences observed in both temperature and frequency sweep analyses may thus be attributed to effects of varying extent of proteolysis, and probably differences in peptide profiles. Results indicated that rheological characteristics of PS and RS cheeses were similar (Fig. 4 and 5). Regardless of differences in extent of proteolysis that characterized the cheeses such differences may be attributed to the differences in peptide profiles.

CONCLUSIONS

DYNAMIC RHEOLOGICAL MEASUREMENTS using a controlled-stress rheometer are suitable for monitoring changes in rheological properties of Cheddar cheese throughout ripening. By using an upper plate with 80-μm teeth, specimen slippage could be avoided and the need for gluing eliminated. Changes in viscoelastic characteristics of Cheddar cheese are affected by overall extent of proteolysis during ripening. However, when comparing such changes in different cheese types, rheological analyses should be evaluated based on overall content of soluble N-containing compounds of different cheese extracts and also on specific peptide profiles as well. Test temperature affected results of rheological analyses, which should be considered in establishing test conditions.

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Reduction of Aflatoxin M_1 from Artificially Contaminated Milk Using Ultrafiltration and Diafiltration

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ABSTRACT

The effect of ultrafiltration-diafiltration (UF-DF) on removal of aflatoxin M_1 (AFM_1) from raw whole, homogenized and acidified milk was studied. Milk was artificially augmented with concentrations of AFM_1 varying from 0.5 to 3.5 μg/L. The removal of AFM_1 was influenced ($p<0.05$) by the initial concentration and the homogenization process. Homogenization and acidification decreased the removal of AFM_1 from milk. The UF-DF process did not result in concentrates within permissible residual levels at any concentration studied. However, reconstituted retentates had residual levels of $AFM_1 < 0.5$ μg/L resulting from milk originally containing up to 2 μg/L.

Key Words: milk, aflatoxin M_1, ultrafiltration, diafiltration

INTRODUCTION

AFLATOXIN M_1 (AFM_1) is a toxic, carcinogenic compound, which may be in milk when cows consume feed contaminated with aflatoxin B_1 (AFB_1). The hepatic microsomal mixed-function oxidase system of the cow converts about 1–2% of AFB_1 into AFM_1, which is excreted into the milk (Price et al., 1985). There is concern about potential ill effects of AFM_1 in milk on public health. Fourteen countries have established maximum levels of AFM_1 permissible in fluid milk varying from 0.05 to 1 μg/L (Van Egmond, 1989). In the U.S., milk containing AFM_1 at > 0.5 μg/L must be removed from interstate commerce (Yousef and Marth, 1987).

One approach to overcome this problem is to eliminate the toxin from milk. Various treatments (physical, chemical or biological) have been applied to contaminated milk for inactivating or removing AFM_1. Pasteurization and sterilization (Purchase et al., 1972), hydrogen peroxide (Applebaum and Marth, 1982a), sulfite and bentonite (Applebaum and Marth, 1982b), and ultraviolet irradiation (Yousef and Marth, 1986) have been evaluated for this purpose. However, there remains a need for an effective method, compatible with practices of the dairy industry.

Ultrafiltration (UF) separates molecules from suspensions or solutions based on size, charge, shape, and affinity for a membrane. Diafiltration (DF) is the application of water to retentates to remove impurities or undesirable compounds. The combination of UF and DF may be ideal for separation of low-molecular-weight contaminants from milk. Kosikowski and Jimenez-Flores (1987) developed a process for antibiotic removal through UF-DF. Because AFM_1 is of low molecular weight (328 daltons) and milk UF is common in many dairy industries, our objective was to assess effects of UF-DF on removal of AFM_1 added to raw whole milk, homogenized and unhomogenized. Our working hypothesis was that removal of AFM_1 could be diminished by the UF-DF process.

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MATERIALS & METHODS

Sample

Raw whole milk was obtained from a dairy farm at the University of Sonora, Mexico, (autumn 1993). The effect of AFM_1 concentration on removal of the toxin was studied with 20-L lots of unhomogenized milk, artificially augmented with AFM_1 (Sigma, St. Louis, MO) at 1, 1.5, 2, 2.5, and 3.5 μg/L. To analyze the effects of homogenization on removal of AFM_1, 20-L lots were first homogenized using a laboratory-scale homogenizer (Model 15M-8TA, Gaulin Co., Wilmington, MA). AFM_1 was then added to the milk at 0.6 and 0.7 μg/L. One UF-DF run was also performed with acidified and unhomogenized milk. Acidification was accomplished by adding a 0.5 M citric acid solution to pH 6.0 and adding AFM_1 at 2.5 μg/L. The aflatoxin was dissolved in methanol before it was added to any milks.

Ultrafiltration-diafiltration

The AFM_1-augmented milk was ultrafiltered at constant temperature (55°C) to two-thirds (3:2), and one-third (3:1) of its original volume in a Romicon pilot-scale hollow-fiber UF unit (Model HFLAB-5, Romicon. Inc., Woburn, MA), equipped with one membrane cartridge of ≈ 4500 cm^2 filtering surface with molecular weight cut-off of 10,000 daltons. Inlet pressure was 1.7 kg/cm^2 and outlet 0.7 kg/cm^2. The experiment was repeated twice for all concentrations of AFM_1 except for 1.5, and 3.5 μg/L which were only run once.

The resulting 3:1 retentate from each lot was washed by adding an equal volume of distilled water (6.6 L) at 55°C. Thereafter, the mixture was ultrafiltered again to 3:1 vol/vol concentration. This diafiltration was repeated three times. A 300-mL sample of each retentate was collected and reconstituted with uncontaminated ultrafiltration milk permeate for further analyses.

AFM_1 analysis

AFM_1 concentration of reconstituted milk samples was measured according to Hansen (1990) in triplicate. A 50-mL portion was mixed with 1g NaCl and centrifuged at 4000 rpm for 5 min. The skim portion was filtered before analysis. A 25-mL aliquot of reconstituted milk was passed through an antibody affinity column to isolate AFM_1 (Aflatest™, Vicam, Somerville, MA). Samples were passed through the column under air pressure (210-350 g/cm^2) supplied by a small aquarium-style air pump. The column was then washed with two 10-mL portions of 10% methanol, and the AFM_1 eluted with 1 mL of 80% methanol. Each eluate was mixed with 1 mL of 0.001% aqueous bromine. Fluorescence was then measured on a TorBex FX-100 Serie 3 (Vicam, Somerville, MA) fluorometer. To assess recovery percentage, a reconstituted standard with 0.25 μg/L AFM_1 with aflatoxin-free skim milk was prepared (Penicillin Assays Inc., Malden, MA) and analyzed in duplicate.

Proximate analysis

The following analyses were performed to determine changes in milk composition during UF-DF: Moisture by microwave procedure (Barbano and De la Valle, 1984); milk fat by the Babcock method; nitrogen by micro-Kjeldahl; and ash by incineration at 700°C. All analyses were performed according to AOAC (1990) methods. Total carbohydrate was determined by difference.

Statistical analysis

Analysis of covariance was performed with 95% confidence limits for determining significant differences in removal rates using methodology outlined by Zar (1985).

Table 1—Effect of ultrafiltration-diafiltration on unhomogenized whole raw milk artificially augmerted with aflatoxin M_1

Initial concentration µg/L[h]	Reconstituted retentate 3:1 µg/L[h]	Reconstituted retentate 1W[g] µg/L[h]	Reconstituted retentate 3W[g] µg/L[h]
0.95 ± 0.03[a]	0.54 ± 0.02[b]	0.43 ± 0.02[c]	0.33 ± 0.02[d]
1.45[e]	0.95	0.75	ND[f]
2.03 ± 0.07[a]	0.98 ± 0.04[b]	0.71 ± 0.11[c]	0.48 ± 0.02[d]
2.41 ± 0.34[a]	1.46 ± 0.05[b]	1.04 ± 0.08[c]	0.72 ± 0.02[d]
3.45[e]	2.1	1.9	1.4

[a-d] Means with the same letter within rows are not significantly different (P<0.05).
[e] One run.
[f] ND = not determined.
[g] 1W = concentrate after 1 wash; 3W = concentrate after three washes.
[h] Average ± standard deviation.

Table 2—Proximate analysis of whole raw milk and reconstituted retentates from ultrafiltration and diafiltration[d]

	Whole raw milk	Reconstituted retentate 3:1	Reconstituted retentate 3W[e]
Total solids	12.80 ± 1.44[a]	12.58 ± 1.66[a]	11.44 ± 2.90[a]
Crude protein[b]	3.47 ± 0.22[a]	3.36 ± 0.64[a]	3.34 ± 1.39[a]
Crude fat	3.21 ± 0.14[a]	3.24 ± 1.05[a]	3.32 ± 0.65[a]
Ash	0.70 ± 0.04[a]	0.72 ± 0.13[a]	0.59 ± 0.17[a]
Lactose[c]	5.42 ± 1.44[a]	5.26 ± 1.58[a]	4.19 ± 1.59[a]

[a] Means with same letter not significantly different (P<0.05).
[b] Crude protein: N × 6.38.
[c] Lactose: by difference.
[d] Average of six runs; Average ± standard deviation (%).
[e] 3W = concentrate after three washes.

Table 3—Effect of ultrafiltration-diafiltration on unhomogenized whole raw milk artificially augmented with aflatoxin M_1

Initial concentration µg/L[g]	Retentate 3:1 µg/L[g]	Retentate 1W[f] µg/L[g]	Retentate 3W[f] µg/L[g]
0.95 ± 0.03[a]	1.64 ± 0.07[b]	1.31 ± 0.07[c]	0.99 ± 0.06[a]
1.45[d]	2.8	2.3	ND[e]
2.03 ± 0.07[a]	2.93 ± 0.11[b]	2.12 ± 0.33[a]	1.43 ± 0.07[c]
2.41 ± 0.39[a]	4.39 ± 0.14[b]	3.11 ± 0.25[c]	2.16 ± 0.07[a]
3.45[d]	6.3	5.7	4.2

[a-c] Means with the same letter within rows are not significantly different (P<0.05).
[d] One run.
[e] ND = not determined.
[f] 1W = concentrate after 1 wash; 3W = concentrate after three washes.
[g] Average ± standard deviation.

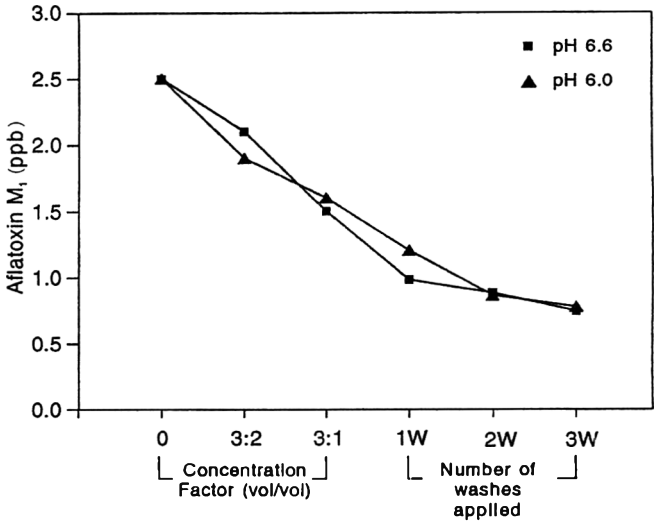

Fig. 1—Effect of acidification on removal pattern of AFM_1.

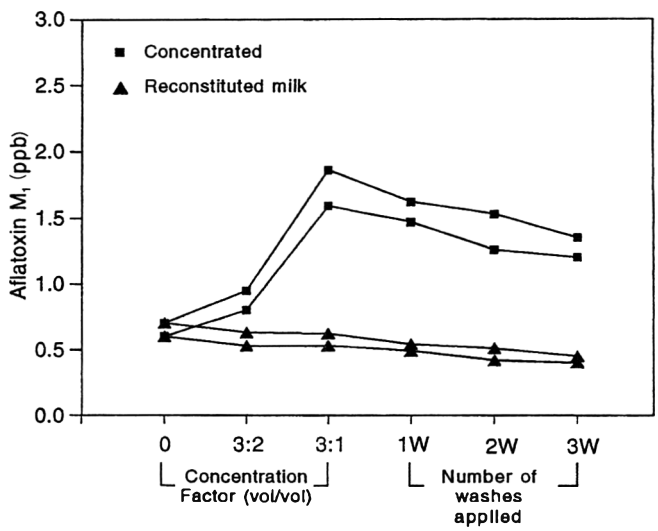

Fig. 2—Effect of ultrafiltration-diafiltration on homogenized whole raw milk contaminated with aflatoxin M_1.

RESULTS & DISCUSSION

Effects of concentration

When results from reconstituted retentates were compared, removals of AFM_1 by ultrafiltration and diafiltration were ($p<0.05$) different based on the initial concentration. Ultrafiltration, on average, removed 43.8% of AFM_1, while diafiltration removed 25.0% of the initial concentration. Overall removal of AFM_1 through both processes was 68.5% (Table 1).

Slope comparison analysis proved that removal of AFM_1 was affected by level of initial concentration during both ultrafiltration and diafiltration. The coefficient of variation in percentage of removal for all runs was < 17%, thus indicating that there was no large increment (%) in removal of AFM_1 when its concentration increased. The higher the initial level of the contaminant the larger the residual concentration remaining in the retentate.

This effect of initial concentration on removal has been reported with other low-molecular-weight toxic compounds like penicillin G, tetracycline and streptomycin from milk (Kosikowski and Jimenez-Flores, 1985). The initial concentration influences removal due to the fractionation of different milk components, which stabilize at specific concentrations and cause fouling of membranes. The addition of water (diafiltration) temporarily disrupts the impregnation of solids into the membrane pores and increases overall process flux.

Starting with milks with artificially added AFM_1 concentrations up to 2 µg/L, the UF-DF process resulted in reconstituted retentates with AFM_1 concentrations lower than the maximum allowable limit (0.5 µg/L). The chemical composition of reconstituted milks was very similar to that of the original milk (Table 2). However, the diafiltration of retentates was reported to produce milk that was not very flavorful (Kosikowski and Jimenez-Flores, 1985).

Applebaum et al. (1982) initially considered that milk contaminated with AFM_1 could be used for manufacture of cheeses. However, they observed that the concentration of AFM_1 increased in the cheese, probably due to casein-binding and the stability of the casein-AFM_1 complex. The same phenomenon probably occurred during the UF process, since the AFM_1 content increased with the concentration factor, the initial level of contamination, and protein content (Table 3).

Ultrafiltration increased the content of AFM_1 in the 3:1 retentate by 71.4%. The percent recovery of the AFM_1 varied from 34 to 88% in the 3:1 retentates. This percentage of rejection was inversely proportional to the initial content of AFM_1. After application of three washes to these concentrates, the initial content of aflatoxin decreased by a small percentage but remained above the maximum allowable limit.

Effect of acidification

Milk acidification was performed in order to partially disrupt the protein fraction (Bastian et al., 1991) with which the AFM_1

is associated (Applebaum et al., 1982). In our study, acidification of milk from pH 6.6 to 6.0 did not influence removal of AFM_1 ($p<0.05$) (Fig. 1). These results confirmed those of Yousef and Marth (1987) who reported stability of AFM_1 in aqueous solutions irradiated with ultraviolet energy in a pH range 3.0–7.0.

Effect of homogenization

Comparison of results from reconstituted retentates showed that removal of AFM_1 from homogenized milk by the ultrafiltration process was not significant. This was contrary to effects of diafiltration (Fig. 2). Removal during ultrafiltration was 11.4% and during diafiltration 23.6%. The combination of the processes removed an average 35% thus the AFM_1 residual levels in reconstituted milks were lower than 0.5 μg/L. However, at least one wash step after ultrafiltration was required, even when the initial concentrations of AFM_1 were low (0.6 and 0.7 μg/L).

Ultrafiltration increased the content of AFM_1 in the 3:1 retentate by 165%. Percent retention was 89% at every concentration. After application of the three washing steps to the concentrates, aflatoxin remained at > 96% of the original concentration (Fig. 2). This concentration effect resulted in retentates with residual levels above the maximum allowable level, even when the initial content of AFM_1 had been low. The ratio of AFM_1 concentration in retentates 3:1 was 1.8:1 in unhomogenized milk and 2.7:1 in homogenized milk.

This retention of AFM_1 in homogenized milk could be due to the fact that the protein retention coefficient is >90% and a large portion of the aflatoxin molecule is bound to the protein fraction. Homogenization stabilizes all milk components in one phase making the protein-AFM_1 association more stable.

The method used to quantify AFM_1 had an efficiency of recovery of 96% and a very slight variation in triplicate runs. The detection limit is 0.05 μg/L. Hansen (1990) reported that results by this fluorometric technique were very similar to those by HPLC.

The application of ultrafiltration-diafiltration processes to reduce levels of AFM_1 contamination in milk have good potential. Many dairy processing plants are utilizing ultrafiltration technology. These plants produce large volumes of fresh permeate with little use. Thus, no additional infrastructure or materials would be needed to clean up and reconstitute contaminated milk. The percent of removal based on reconstituted retentates could be notable and comparable to proposed methods which require more costly equipment.

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Ms received 7/27/94; revised 12/10/94; accepted 1/21/95.

Erratum Notice

•*J. Food Sci.* (Vol. 60, No. 2, 1995) pages 416–419—ε-(γ-Glutamyl)lysine crosslink distribution in foods as determined by improved method by H. Sakamoto, Y. Kumazawa, H. Kawajiri, and M. Motoki. Dr. Kumazawa advises errors were detected on page 417 in Materials & Methods under the Preliminary chromatographic fractionation section as follows: The elution time should read 10.2 min not 12.5 as shown on lines 7 and 14. Please correct accordingly.

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INSTITUTE OF FOOD TECHNOLOGISTS

THE SOCIETY FOR FOOD SCIENCE AND TECHNOLOGY

Guidelines for the Preparation and Review of Research Papers Reporting Food Engineering Data

IFT Food Engineering Division

The Food Engineering Division of the Institute of Food Technologists has prepared the following guidelines for use by authors in preparing food engineering-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.

The guidelines, originally prepared in 1989, have been revised by R.Y. Ofoli. The guidelines were originally assembled by a committee consisting of R.Y. Ofoli, M.A. Rao, R.P. Singh, and J.F. Steffe and represent the combined inputs of many Food Engineering Division members. In addition, the following important references were consulted by the committee in preparing the nomenclature list:

AIChE. 1979. Letter symbols for chemical engineering. Am. Inst. of Chem. Eng., New York.

Dealy, J.M. 1984. Official nomenclature for material functions describing the response of a viscoelastic fluid to various shearing and extensional deformations. J. Rheol. 28(3): 181.

Suggested Format

I. Abstract

II. Introduction
- A. Statement of problem, including relevance
- B. Brief review of the literature
- C. Specific objectives of the study

III. Theoretical Considerations
- A. Mathematical expressions appropriate to problem statement
- B. Derivation of theoretical relationships (if not available in published literature)

IV. Materials & Methods (Experimental Procedures)
- A. Description of experimental material (including manufacturer's specifications) and procedures
- B. Description of computer program (if used)
  1. Flow diagram
  2. Language used in programming
  3. Type of computer used and execution time
- C. Statistical design

V. Results & Discussion
- A. Theoretical derivations
- B. Experiments
- C. Verification of theoretical results
- D. Verification of computer results

VI. Conclusions (should not be just a summary statement)

VII. Nomenclature (all symbols and units used in the paper should be defined)

VIII. References (see Style Guide for Research Papers, *J. Food Sci.* 59: 1367-1370 (1995)

Suggested Nomenclature

1. General Concepts

Term	Symbol	Unit or definition
Acceleration	a	ms^{-2}
Acceleration of gravity	g	ms^{-2}
Base of natural logarithms	e	
Coefficient	C	
Difference, finite	Δ	
Differential operator, total	d	
Differential operator, partial	∂	
Efficiency	η	
Energy, dimension of	E	J, N m
Enthalpy	H	J
Entropy	S	J/K
Force	F	N
Gas constant, universal	R	To distinguish use R_o
Gibbs free energy	G,F	G = H − TS, J
Heat	Q	J
Helmholtz free energy	A	A = U − TS, J
Internal energy	U	J
Mass, dimension of	m	kg
Mechanical equivalent of heat	J	J, N m
Moment of inertia	I	m^4
Newton law of motion, conversion factor	g_c	
Number of moles	mol	
Pressure	p	Pa, bar
Quantity, in general	Q	
Ratio, in general	R	
Resistance	R	Ω
Temperature, dimensionless	Θ	
Temperature, absolute	T	K
Temperature, in general	T	°C
Time, in general	t	s, h
Work	W	J
Electric current	I	A
Electric potential	V	V
Capacitance	C	F

2. Geometrical Concepts

Term	Symbol	Unit or definition
Linear dimension		
Breadth	b	m
Diameter	D	m
Distance along path	s, x	m
Height above datum plane	Z	m
Height equivalent	H	m
Hydraulic radius	r_H	m
Lateral distance from datum plane	Y	m
Length	L	m
Longitudinal distance from datum plane	X	m
Mean free path	λ	m
Radius	r	m
Thickness, in general	B	m
Thickness of film	B_f	m
Wavelength	λ	m
Area		
In general	A	m^2
Cross section	A_s	m^2
Projected	A_p	m^2
Surface per unit mass	A_w, s	m^2/kg
Surface per unit volume	A_s, a	m^2/m^3
Volume		
In general	V	m^3
Void fraction	ϵ	
Humid volume	V_H	m^3/kg dry air
Angle		
In x, y plane	α	
In y, z plane	ϕ	
In z, x plane	θ	
Solid angle	ω	
Other		
Particle-shape factor	ϕ_s	

3. Intensive Properties

Term	Symbol	Unit or definition
Absorptivity for radiation	α	
Activity	a	mol/m^3
Activity coefficient, molal basis	γ	
Coefficient of expansion, linear	α	m/(m K)
Coefficient of expansion, volumetric	β	$m^3/(m^3K)$
Compressibility factor	z	z = p V/RT
Density	ρ	kg/m^3
Diffusivity, molecular, volumetric	D_v, δ	$m^3/(s\ m)$, m^2/s
Diffusivity, thermal	α	$\alpha=k/(\rho C_p)$, m^2/s
Emissivity ratio for radiation	ϵ	
Enthalpy, per mole	H	J/kmol
Entropy, per mole	S	J/(kmol K)
Fugacity	f	Pa, bar
Gibbs free energy, per mole	G, F	Jkmol
Helmholtz free energy, per mole	A	J/kmol
Humid heat	c_s	J/(kg dry air K)
Internal energy	U	J/kmol
Latent heat	H_L	J/kg
Molecular weight	MW	kg/kmol
Reflectivity for radiation	ρ	
Heat capacity	C	J/(kg K)
Heat capacity, at constant pressure	C_p	J/(kg K)
Heat capacity, at constant volume	C_v	J/(kg K)
Surface tension	σ	N/m
Thermal conductivity	k	(J m)/(s m^2 K)
Transmissivity of radiation	τ	
Vapor pressure	p*	Pa, bar
Specific volume	V	m^3/kmol

4. Symbols for Concentration

Term	Symbol	Unit or definition
Concentration, mass or moles per unit volume	C	kg/m^3, $kmol/m^3$
Fraction, cumulative beyond a given size	ϕ	
Fraction, by volume	x_v	
Fraction, by weight	x_w	
Humidity	H	kg H_2O/kg dry air
Humidity at saturation	H_s	kg H_2O/kg dry air
Humidity at wet-bulb temperature	H_w	kg H_2O/kg dry air
Humidity at adiabatic saturation	H_a	kg H_2O/kg dry air
Mass concentration of particles	C_p	kg/m^3
Moisture content, total water to bone-dry stock	X_{db}	kg H_2O/kg dry stock
Moisture content, equilibrium water to bone-dry stock	X_e	kg H_2O/kg dry stock
Moisture content, free water to bone-dry stock	X	kg H_2O/kg dry stock

Term	Symbol	Unit or definition
Mole or mass fraction, in heavy or extract phase	x	
Mole or mass fraction, in light or raffinate phase	y	
Mole or mass ratio, in heavy or extract phase	X	
Mole or mass ratio, in light or raffinate phase	Y	
Number density	n_p	number/m^3
Phase equilibrium ratio	K	K = y/x
Relative distribution of two components:		
Between two phases in equilibrium	α	$\alpha = K_i/K_j$
Between successive stages	β	$\beta_n = (y_i/y_j)_n/(x_j/x_i)_{n+1}$
Relative humidity	RH	
Slope of equilibrium curve	m	m = dy/dx

5. Symbols for Rate Concepts

Term	Symbol	Unit or definition
Quantity per unit time, in general	q	
Angular velocity	ω	
Feed rate	F	kg/s, kmol/s
Frequency	f, N_f	Hz
Friction velocity	u^*	$u^* = (\sigma\rho)^{1/2}$, m/s
Heat transfer rate	q	J/s
Heavy or extract phase rate	L	kg/s, kmol/s
Heavy or extract product rate	B	kg/s, kmol/s
Light or raffinate phase rate	V	kg/s, kmol/s
Light or raffinate product rate	D	kg/s, kmol/s
Mass flow rate	w, m	kg/s, kg/h
Molal transfer rate	N	kmol/s
Power	P	W
Revolutions per unit time	n	rpm, rps
Velocity, in general	u	m/s
Velocity, longitudinal (x)	u	m/s
Velocity, lateral (y)	v	m/s
Velocity, normal (z)	w	m/s
Volumetric flow rate	$\dot{V}$	m^3/s, m^3/h
Quantity per unit time, unit area		
Emissive power, total	W	W/m^2
Mass velocity, average	G	$G = w/A_s$, kg/(s m^2)
Vapor or light phase	G	kg/(s m^2)
Liquid or heavy phase	L	kg/(s m^2)
Molar flux	J	kmol/s
Mass flux	j	kg/s
Radiation, intensity of	I	W/m^2
Velocity, nominal, basis total cross-section of packed vessel	v_s	m/s
Quantity reacted per unit time, reactor volume	N_R	kmol/(s m^3)
Space velocity, volumetric	Λ	$m^3/(s\ m^3)$
Quantity per unit time, unit area, unit driving force, in general	k	
Eddy diffusivity	δ_E	m^2/s
Eddy viscosity	υ_E	kg/(ms)
Heat transfer coefficient	h	$W/(m^2K)$
Individual		
Overall	U	$W/(m^2K)$
Mass transfer coefficient	k	kmol/(s m^2)
Individual		(driving force), where driving force is kmol/m3, bar, or mole fraction
Gas film	k_G	
Liquid film	k_L	
Overall	K	
Gas film basis	K_G	
Liquid film basis	K_L	
Stefan-Boltzmann constant	σ	5.6703×10^{-8} $W/(m^2K^4)$

6. Symbols for Rheological Measurements

Term	Symbol	Unit or definition
Steady simple shear		
Direction of flow	x	m
Direction of velocity gradient	y	m
Neutral direction	z	m
Shear stress	σ	Pa
Shear strain	γ	
Shear rate	$\dot{\gamma}$	s^{-1}
Apparent viscosity	η	Pa s
Effective viscosity	μ_e	Pa s
Newtonian viscosity	μ	Pa s
First normal stress function	N_1	Pa
Second normal stress function	N_2	Pa
First normal stress coefficient	Ψ_1	Pa s^2
Second normal stress coefficient	Ψ_2	Pa s^2
Limiting viscosity at zero shear rate	η_0	Pa s
Limiting viscosity at infinite shear rate	η_∞	Pa s
Viscosity of solvent or of continuous medium	η_s	Pa s
Relative viscosity (η/η_σ)	η_r	
Specific viscosity ($\eta_r - 1$)	η_{sp}	
Intrinsic viscosity	η	m^3/kg
Linear viscoelasticity		
Simple shear		
Shear strain	γ	
Shear modulus (modulus of rigidity)	G	Pa
Shear relaxation modulus	G(t)	Pa
Shear compliance	J	Pa^{-1}
Shear creep compliance	J(t)	Pa^{-1}
Equilibrium shear compliance	J_e	Pa^{-1}
Steady-state shear compliance	J_s°	Pa^{-1}
Complex viscosity	$\eta^*(\omega)$	Pa s
Dynamic viscosity	$\eta'(\omega)$	Pa s
Out-of-phase component of η^*	$\eta''(\omega)$	Pa s
Complex shear modulus	$G^*(\omega)$	Pa
Shear storage modulus	$G'(\omega)$	Pa
Shear loss modulus	$G''(\omega)$	Pa
Complex shear compliance	$J^*(\omega)$	Pa^{-1}
Shear storage compliance	$J'(\omega)$	Pa^{-1}
Shear loss compliance	$J''(\omega)$	Pa^{-1}
Tensile extension		
Hencky strain	ϵ	
Young's modulus	E	Pa
Tensile relaxation modulus	E(t)	Pa
Tensile compliance	D	Pa^{-1}
Tensile creep compliance	D(t)	Pa^{-1}

7. Symbols for Thermal Process Design

Term	Symbol	Unit or definition
Decimal reduction time, time required for number or concentration of spores to be reduced by a factor of ten at given temperature T	D_T	min, s
Heating rate parameter; time in minutes required for the difference between retort temperature and food temperature to decrease or increase by a factor of ten; the inverse slope of a straight-line portion of the heat penetration curve	f	min
f value in a cooling process	f_c	min
f value in a heating process that is characterized by a single straight-line segment or the first segment of a broken heat penetration curve	f_h	min
f value for the second straight-line segment of a broken heat penetration curve	f_2	min
Amount of time required to destroy a given percentage of microorganisms at a given temperature	F	min
Sterilization value; equivalent exposure time in minutes at the reference temperature T_{ref}	$F^z_{T_{ref}}$	min
received by the slowest-heating zone in the container with regard to the destruction of a microorganism whose thermal resistance is characterized by z min. Sterilization value for a reference temperature of 121.1°C and a z value of 10C°	F_0	min
Difference between retort temperature and container temperature measured at the slowest-heating zone at the end of the heating process	g	°C
g at the break (change in slope) of the heat penetration curve	g_{bh}	°C
Heating lag factor defined as $(T_r - T_A)/(T_r - T_i)$ where T_A is the pseudo initial temperature of the container equal to the intercept of the straight-line heating curve and the axis defining the effective beginning of the heating period	j	
Lag factor for the cooling phase	j_c	
Bessel function of first kind or order zero	j_0	
Bessel function of first kind or order one	j_1	
Difference between cooling-water temperature and temperature attained at the slowest-heating zone in container at the end of the heating process	m	°C
Time measured from the beginning of heating	t	min, s
Time in a thermal process corresponding to the end of heating phase and initiation of cooling phase	t_B	min, s
	t_B	min, s
Temperature of the food at the slowest-heating zone in the container	T	°C
Uniform initial temperature of the container contents	T_i	°C
Exposure temperature for the container; retort temperature	T_r	°C
Sterilization value for a reference temperature equal to retort temperature	U	min
Time in minutes at which the break (change in slope) of the broken heat penetration curve occurs	X_{bh}	min
A value, characteristic of a microorganism, which measures the change in the death rate with respect to a change in temperature; numerically equal to the number of Celsius degrees required to change by a factor of ten the time required to kill an organism at a specified temperature; dimension of temperature degrees	z	C°

8. Symbols for Membrane Processing

Term	Symbol	Unit or definition	Term	Symbol	Unit or definition
Flux, the volume of permeate per unit membrane area per unit time	J	L/m^2h	Diffusion coefficient	D	m^2/s
			Flow rate through module (recycling rate)	Q	L/min
Transmembrane pressure	ΔP_T	$\Delta P_T = (P_i+P_o)/2 - P_p$, kPa	Linear velocity	V	m/s
			Fouling index in first-order model	b	
Inlet pressure	P_i	kPa			
Outlet pressure	P_o	kPa	Rejection	R	$R = 1 - (C_p/C_r)$ where C_p is concentration of species in permeate and C_r is concentration in retentate
Permeate back pressure	P_p	kPa			
Pressure drop along module or across pump	ΔP	kPa			
Effective membrane surface area	A	m^2			
Mass transfer coefficient used in film theory	k	L/m^2 hr			