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Research Notes

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Volume 51: Number 6

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Memo *FROM THE SCIENTIFIC EDITOR*

Scientific knowledge has made a quantum leap forward in the past quarter of a century, powered, principally, by the development of highly sensitive, sophisticated instrumentation. These instruments have permitted us to find the answers to yesterday's problems, and to postulate even more incisive questions for tomorrow.

Equally impressive in this surge of knowledge has been the development of new concepts that are leading us closer to understanding the basic nature—chemically and physically—of the universe.

Food science, although behind in the advances made in the other disciplines, is now beginning to show the effects of developments in chemistry, biochemistry, microbiology, and engineering. This is observed not only in the research laboratories but also in the processing plants. DNA modification, for example, has been practised for many years in developing desirable characteristics in seed stock and breeding lines, but gene manipulation achieves results more specifically and quicker. Coupled with the newer techniques in biotechnology it may be possible to develop new, or improved, sources of enzymes, or flavors, or even food products. Sophisticated computer programs permit modeling of various engineering processes, obtaining results much more quickly than possible by actual experimentation, especially if a number of variable parameters are involved.

Manuscripts are now being submitted to the *Journal of Food Science* that reflect this newer technology. Some of them have not been accepted because it appeared that they were more appropriate for one of the more specialized publications or that they were not directly related to the traditional concept of food science. However, since food science is itself a multi-discipline discipline the *Journal* editorial staff and reviewers will have to reevaluate their standards to accommodate the newer technologies.

We should like to hear the readers views on their fields of interests and on their concept of food science of the future so that we can identify the manuscripts that will anticipate these developments.

We would appreciate your comments on how the *Journal of Food Science* is serving—or not serving—your needs.

To make possible timely publication of a journal of this size requires the dedicated efforts of a number of people. I wish to thank Anne Haubrich, in my office, Dr. Stan Kazeniac and Anna May Schenck, JFS Associate Scientific Editors, for the time and care they have devoted. John Klis (Director of Publications), Barney Schukraft (Managing Editor), and Gladys Anderson (Editorial Department Secretary) of the IFT Publications Office have been very helpful and I am pleased to acknowledge their support and assistance.

The Editorial Board has also been very helpful, and I wish to express my thanks to the retiring Board members and welcome the new members as they begin their three-year term.

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The reviewers are the backbone of the Journal. These are a group of dedicated scientists who, in spite of their busy schedules as researchers or teachers, read and provide helpful comments for the manuscripts that have been submitted. To these reviewers, listed below, I express my thanks and appreciation, and to anyone whom I have inadvertently omitted, I offer my apologies.

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Kinetics of Decomposition of Aspartame Hydrochloride (Usal) in Aqueous Solutions

M. PRUDEL, E. DAVÍDKOVÁ, J. DAVÍDEK, and M. KMÍNEK

ABSTRACT

A scheme of aspartame hydrochloride (Usal) decomposition in relation to the pH and temperature which takes into account the possibility of phenylalanyl-aspartic acid dipeptide formation is suggested and experimentally confirmed. Aspartyl-phenylalanine and diketopiperazine were found to be the main decomposition products. The concentration of diketopiperazine increases and that of aspartyl-phenylalanine decreases with increasing pH. At pH 2.9 less aspartyl-phenylalanine and more diketopiperazine is formed with increase in temperature; twice the concentration of phenylalanine methyl ester was found at 80° and 90°C when 50% of the Usal present in the medium was decomposed, as compared to that determined at 25° and 40°C. The distribution of the remaining products remained constant over the entire range measured.

INTRODUCTION

IT IS WELL KNOWN that both the aspartame (l-methyl-N-L-α-aspartyl-L-phenylalanine) and its hydrochloride (prepared in Czechoslovakia under the trade mark Usal), i.e. dipeptidic sweeteners composed of two amino acids - methyl ester of phenylalanine and aspartic acid, exhibit only limited stabilities in aqueous solutions (Scott, 1974; Prudel and Davídková, 1981). The decomposition of this sweetener corresponds to a first order reaction and its stability is significantly affected by the pH value and temperature of the medium. The optimal pH range from the point of view of stability, ranges between 2.5 and 5.0; lower temperatures being more favourable.

Aspartame decomposition products were first identified by Furda et al. (1975) who applied gas chromatography and proved that dipeptide aspartyl-phenylalanine resulted after splitting off methanol, aspartyl-phenylalanine then either hydrolyzes into individual amino acids or the 3-carboxymethyl-6-benzyl-2,5-dioxopiperazine (diketopiperazine) may form by cyclization. The hydrolysis of aspartame into aspartic acid and phenylalanine methyl ester had also been proved. Boehm and Bada (1984) investigated the racemization kinetics of aspartic acid and phenylalanine in solutions of aspartame heated at 100°C and pH 4 and 7.

The purpose of this study was to investigate the kinetics of decomposition of aspartame hydrochloride in aqueous solutions in relation to pH and temperature.

MATERIALS & METHODS

THE FORMATION of Usal-aspartame hydrochloride (α-L-aspartyl-L-phenylalanine methyl ester hydrochloride (Research Institute for Pharmacy & Biochemistry, Prague, CSSR) decomposition products were investigated at 25 °C in Britton-Robinson buffers (pH 1.1, 1.8, 2.5, 2.9, 3.9, 6.2, 6.8, and 7.1). The effect of temperature was followed at pH 2.9 at 25°, 40°, 60°, 70°, 80°, and 90°C. The solutions tested contained 5 mg Usal·mL^{-1} and were preserved by toluene addition (0.1 μL·mL^{-1}). The diketopiperazine determination was also carried out in two newly developed soft drinks sweetened with fructose and Usal (DIA TONIC and DIA EXOTIC, dry matter refractometrically 1%, Usal concentration 300 mg·L^{-1}, pH 3.1) (Davídková et al., 1983; Prudel and Davídková, 1985) at 7 ± 2° and 20 ± 2°C. The individual products were determined by HPLC (Spectra Physics, Santa Clara, USA) (Prudel and Davídková, 1983, 1985). A stainless steel column (250 × 6 mm) packed with SEPARON SI C-18 (Laboratorní přístroje, Prague, CSSR) and elution mixture 0.5M sodium dihydrogen phosphate (pH adjusted to 2.1 with phosphoric acid) and methanol (85:15, v/v) were used. The flow rate was 2 mL·min^{-1} up to the 30th min and 4 mL·min^{-1} up to the 45th min. The column was used at room temperature. Samples were injected by means of a 10 μL sample loop. Spectrometric detection at 200 nm was applied.

The mass balance was carried out by means of the calibration graphs determined for the individual standard substances (Prudel and Davídková, 1985). The values in the models and in the graphs are expressed in molar equivalents (the initial Usal concentration is taken as 100%).

The rate constants of individual reactions according to the expected course of decomposition scheme (Fig. 1) were calculated by the following series of differential equations:

Model 1:

$$\frac{d[A]}{dt} = -k_1[A] - k_4[A]$$

$$\frac{d[B]}{dt} = k_1[A] + k_3[C] - k_2[B] - k_5[B]$$

$$\frac{d[C]}{dt} = k_2[B] + k_8[F] - k_3[C] - k_7[C]$$

$$\frac{d[D]}{dt} = k_4[A] - k_6[D]$$

$$\frac{d[E]}{dt} = k_6[D] + k_5[B] + k_9[F]$$

$$\frac{d[F]}{dt} = k_7[C] - k_8[F] - k_9[F]$$

Model 2:

$$\frac{d[A]}{dt} = -k_1[A]$$

$$\frac{d[B]}{dt} = k_1[A] - k_2[B] + k_3[C]$$

$$\frac{d[C]}{dt} = k_2[B] - k_3[C]$$

$$t = 0 : [A] = [A]_o, [B] = [B]_o, [C] = [C]_o, [D] = [D]_o,$$

$$[E] = [E]_o, [F] = [F]_o$$

The differential equations were solved by the OPTIMAL program (Varadínek and Burianec, 1978) according to the Rosenbrock method (Rosenbrock and Storey, 1966; Himmelblau, 1972) on a ICL-4-72 computer (ICL, Kidsgrove, UK). The optimization criterion was least squares—the minimization of the sum of squared deviations between the experimental and the calculated (according to the model mechanism) values.

Authors Prudel and Davídková are affiliated with the Research Institute of Food Industry, Prague 5, Na bělidle 21, PSČ 150 38, Czechoslovakia. Authors Davídek and Kminek are affiliated with the Dept. of Food Chemistry, Faculty of Food & Biochemical Technology, Prague Institute of Chemical Technology, Prague 6, Suchbátarova 3, PSČ 166 28.

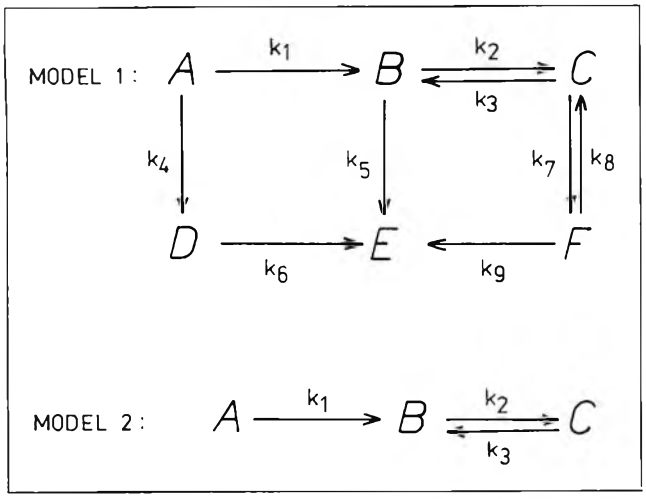

Fig. 1—Proposed course of Usal decomposition. A = aspartyl-phenylalanine methyl ester (Usal); B = aspartyl-phenylalanine; C = diketopiperazine; D = phenylalanine methyl ester; E = phenylalanine; F = phenylalanyl-aspartic acid.

RESULTS & DISCUSSION

THE COURSE of aspartame and Usal decomposition corresponds to a first order reaction (Scott, 1974; Prudel and Davídková, 1981). The mathematical model of Usal decomposition products formation is based on the assumption that all reactions encountered in the model correspond to the first order reaction kinetics. The good agreement between the measured and calculated values are shown in Fig. 2–4.

The calculated values of individual rate constants for pH range 1.1 to 7.1 at 25 °C are given in Table 1. The system in question comprises both acid- and base-catalyzed reactions, the rate constant logarithms of which can be expressed as functions of pH value (Fig. 5–8). The pH change of constant k_1 (i.e. the reaction in which Usal is hydrolyzed to methanol and aspartyl-phenylalanine) is strongly dependent on the concentration of hydrogen(hydronium) and hydroxyl ions that are mutually bonded (Fig. 5). Hence, the relation between the rate constant and the pH value can be expressed by Eq. (1) (Jungers et al., 1958):

$$k = k_o + k_{H^+} \cdot [H_3O^+] + k_{OH^-} \cdot K_v \frac{1}{[H_3O^+]} \quad (1)$$

K_v being the ionic product of water ($K_v = [H_3O^+] \cdot [OH^-] = 1.0.10^{-14}$ mol^2·L^{-2} at 25 °C), k_o being the rate constant of the "spontaneous" reaction ($k_o = kH_2O \cdot [H_2O]$). The constants in Eq. (1) for k_1 were calculated and they are as follows:

$k_o = 2.2 \cdot 10^{-5}$, ks^{-1}, $k_{H^+} = 8.6 \cdot 10^{-3}$ L · mol · ks^{-1}, and $k_{OH^-} = 1.1 \cdot 10^5$ L · $mol^{-1} \cdot ks^{-1}$.

Eq. (1) describes three competitive reactions, each determines the course of reaction in a definite pH range and is not influenced by other conversions. In the range bounded by the segment with negative slope the catalysis is exhibited entirely by H^+ ion; the horizontal segment corresponds to the spontaneous reaction; the segment with positive value of slope is related to catalysis by OH^- ion. In Fig. 5 the values calculated from Eq. (1) are compared with the experimental values. Close agreement between the calculated and observed values is obvious.

The course of the rate constants k_2 and k_8, i.e. constants

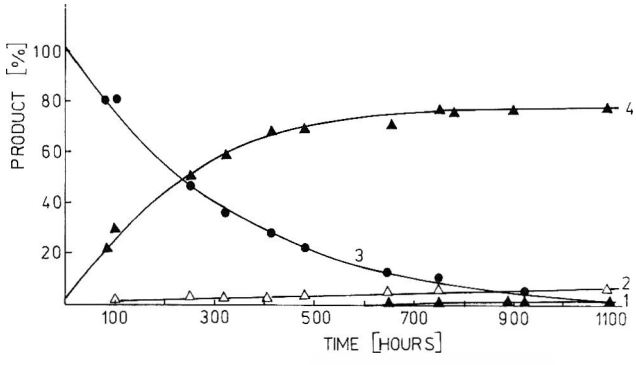

Fig. 2—Course of Usal decomposition in buffer pH 1.1 at 25°C. 1 = phenylalanyl-aspartic acid; 2 = diketopiperazine; 3 = Usal; 4 = aspartyl-phenylalanine.

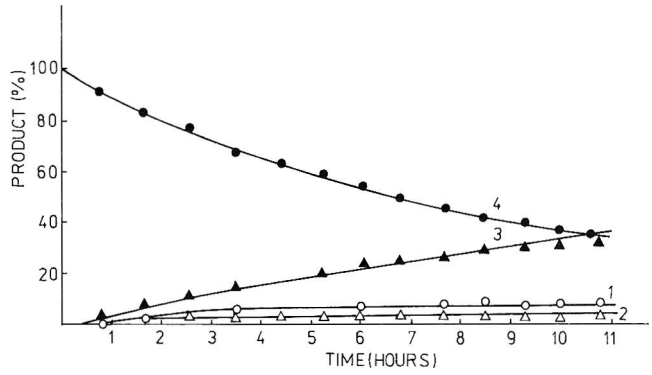

Fig. 3—Course of Usal decomposition in buffer pH 2.9 at 90°C. 1 = aspartyl-phenylalanine (like in phenylalanine); 2 = phenylalanine methyl ester; 3 = diketopiperazine; 4 = Usal.

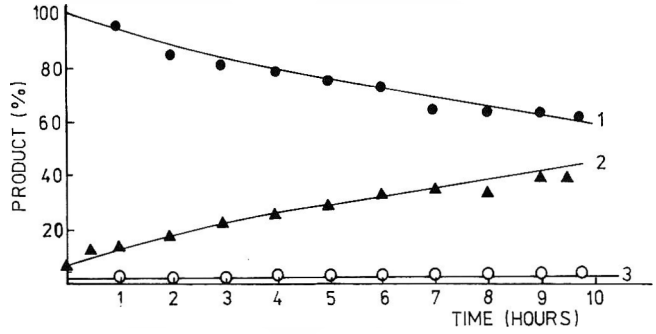

Fig. 4—Course of Usal decomposition in buffer pH 7.1 at 25°C. 1 = Usal; 2 = diketopiperazine; 3 = aspartyl-phenyl-alanine.

Table 1—Measured rate constants in relation to pH value at 25°C

	Rate constants (ks^{-1})								
pH	k_1	k_2	k_3	k_4	k_5	k_6	k_7	k_8	k_9
1.1	8.3×10^{-4}	1.4×10^{-4}	9.7×10^{-4}	6.7×10^{-6}	3.9×10^{-6}	1.2×10^{-5}	7.8×10^{-4}	1.1×10^{-3}	2.0×10^{-3}
1.8	1.6×10^{-4}	6.1×10^{-4}	1.5×10^{-3}	4.4×10^{-5}	2.0×10^{-5}	1.6×10^{-5}	5.6×10^{-4}	1.0×10^{-3}	1.2×10^{-4}
2.5	5.8×10^{-5}	8.1×10^{-4}	1.0×10^{-3}	5.3×10^{-6}	7.8×10^{-6}	7.5×10^{-5}	5.6×10^{-5}	6.7×10^{-3}	1.3×10^{-4}
2.9	4.7×10^{-5}	8.6×10^{-4}	8.3×10^{-4}	4.2×10^{-6}	6.1×10^{-6}	5.3×10^{-5}	9.4×10^{-6}	1.1×10^{-2}	2.3×10^{-5}
3.9	2.8×10^{-5}	9.2×10^{-4}	8.3×10^{-4}	1.7×10^{-6}	5.3×10^{-7}	7.5×10^{-5}	1.7×10^{-5}	1.1×10^{-2}	1.6×10^{-6}
6.2	7.2×10^{-4}	1.6×10^{-1}	1.1×10^{-1}						
6.8	1.0×10^{-2}	6.1×10^{-1}	8.6×10^{-2}						
7.1	1.5×10^{-2}	2.8	2.5×10^{-1}						

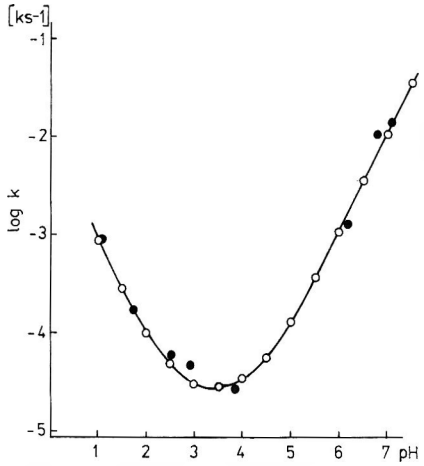

Fig. 5—Relation between the rate constant k_1 and the pH value (25°C). ○ = values calculated from the Eq. (1); ● = measured values.

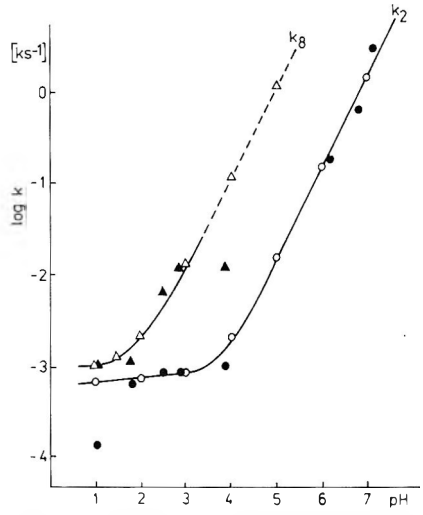

Fig. 6—Relation between the rate constants k_2 and k_8 and the pH value (25°C). △, ○ = values calculated from the Eq. (2); ▲, ● = measured values.

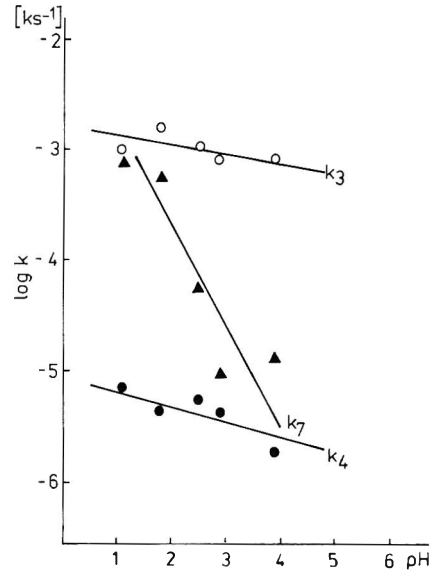

Fig. 7—Relation between the rate constants k_3, k_4 and k_7 and the pH value (25°C).

determining cyclization rates of the dipeptides aspartyl-phenylalanine and phenylalanyl-aspartic acid into diketopiperazine, is dependent on pH (Fig. 6). At lower pH values (for k_2 approx. up to pH 4), the rate of the processes is determined by the constant of "spontaneous" reaction k_o. The catalysis by hydroxyl ions begin to predominate above pH 4. The dependence of k_2 and k_8 on pH can be expressed by the following equation:

$$k = k_o + k_{OH^-} \cdot [OH^-] = k_o + k_{OH^-} \cdot K_V \cdot \frac{1}{[H_3O^+]} \quad (2)$$

The values of the constants k_o and k_{OH^-} in Eq. (2) were calculated as in Eq. (1). The values of the constants k_o and k_{OH^-} for k_2 are as follows; $k_o = 6.9 \cdot 10^{-4}$ ks^{-1} and $k_{OH^-} = 1.4 \cdot 10^7$ L · mol^{-1} · ks^{-1}. The agreement of the calculated and experimental values was satisfactory in this case as well (Fig. 6). Values $k_o = 9.4 \cdot 10^{-4}$ ks^{-1} and $k_{OH^-} = 1.1 \cdot 10^9$ L · mol^{-1} · ks^{-1} were calculated for k_8. However, the correspondence between the calculated and measured values was much worse in the latter case, probably owing to the smaller number of the measured and calculated data (at pH values above 6 the decomposition proceeded according to model 2 (Fig. 1)).

The courses of the remaining rate constants that determine the rate of splitting of the bond between aspartic acid and phenylalanine in dipeptides or in diketopiperazine (k_3, k_4, k_5, k_7, and k_9) are shown in relation to pH (Fig. 7 and 8). They can be expressed by Eq. (3):

$$k = G_A \cdot [H_3O^+]^\alpha \text{ or } \log k = \log G_A - \alpha \text{ pH} \quad (3)$$

The course of k_6 determining the hydrolysis rate of phenylalanine methyl ester to methanol and phenylalanine can be expressed by Eq. (4):

$$k = G_B \cdot K_v \frac{1}{[H_3O^+]^\beta} \text{ or } \log k = \log G_B \cdot K_v + \beta \text{ pH} \quad (4)$$

G_A and G_B being the proportionality constants and α and β the constant exponents similar to those in relations describing the general catalysis by acids and bases (Jungers et al., 1958). The values of the proportionality constants and of the constant exponents are given in Table 2. They represent reactions catalyzed by hydrogen ions (acids) in all cases except that of k_6,

Table 2—Values of proportionality constants and constant exponents for the equation giving the relation between the rate constants and the pH

Rate constant (ks^{-1})	k_3	k_4	k_7	k_9
G_A	1.6×10^{-3}	8.9×10^{-6}	1.4×10^{-2}	3.1×10^{-2}
α	0.1	0.15	0.9	1.0

for k_6: $G_B = 5.3 \times 10^8$, B = 0.3

where the catalysis occurs as a result of the effect of hydroxyl ions (bases). Some of the larger dispersions of the measured values (e.g. for k_5, Fig. 8) are probably caused by a summation of the errors occurring both during the analysis and during the mathematical treatment of this complex kinetic model. The corresponding values of the constants G_A and α for k_5 are, therefore, not given in Table 2.

The change in the reaction velocity and in the rate constant caused by temperature effect represents another characteristic property of chemical reactions. The temperature dependence of the rate constants was studied at pH 2.9 (i.e. at the values corresponding roughly to that of soft drinks in which Usal could be most favorably applied), and is shown in Fig. 9 and 10.

The relations represent the logarithmic transformation of the Arrhenius equation:

$$k = k_o \cdot \exp(-E/RT)$$

where k_o is the frequency factor and E is the activation energy. Hence, it follows that the rate constant increases exponentially with the temperature. The equation thus enables the reaction rate to be determined at any temperature of the range studied, and by means of extrapolation, even at temperatures where it was not measured.

Figure 9 shows that the temperature relation of rate constants k_1–k_4 obeyed the Arrhenius equation. A larger dispersion of the values obtained was obvious for k_5–k_7 (Fig. 9 and 10). The dispersion of the values k_8 and k_9 was so large that fitting them to the Arrhenius equation would not be meaningful (Jungers et al., 1958). The rate constants are given in Table 3.

The comparison of the activation energies of individual reactions, or reactions of the same type, possessed the same activation energies as those found in the ester hydrolysis of Usal and the methyl group in phenylalanine methyl ester (E_1 and E_6), and the hydrolysis of the peptide bond between the

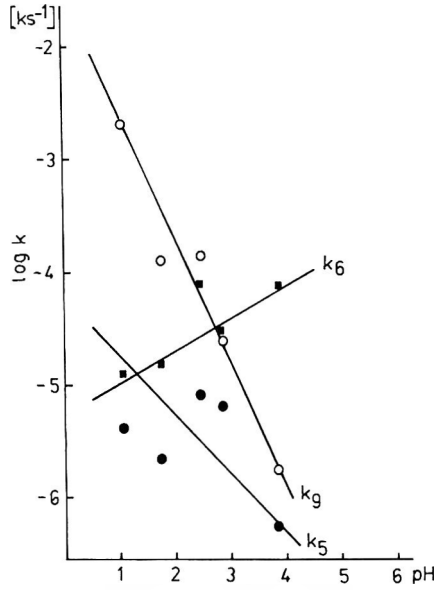

Fig. 8—Relation between the rate constants k_5, k_6 and k_9 and the pH value (25°C).

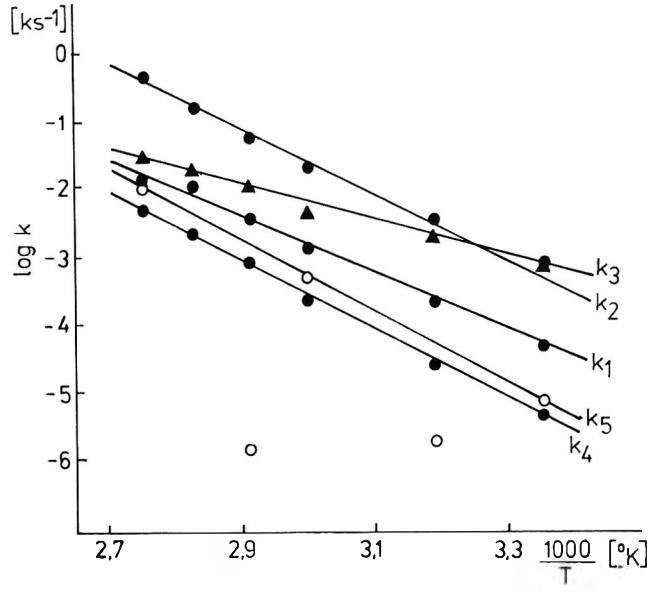

Fig. 9—Relation between the rate constants k_1–k_5 and the temperature (pH 2.9).

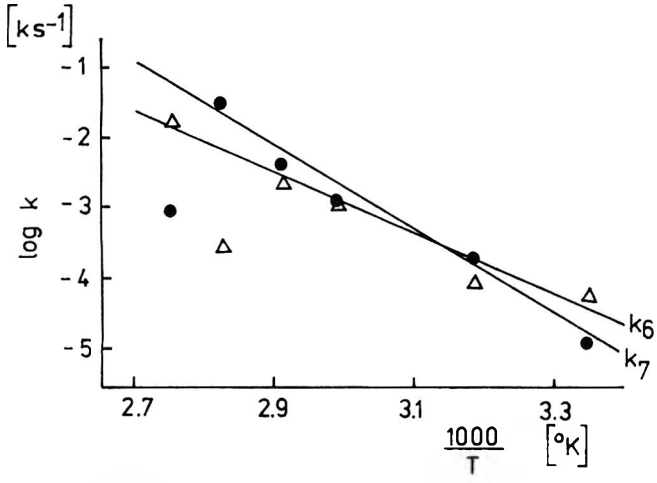

Fig. 10—Relation between the rate constants k_6 and k_7 and the temperature (pH 2.9).

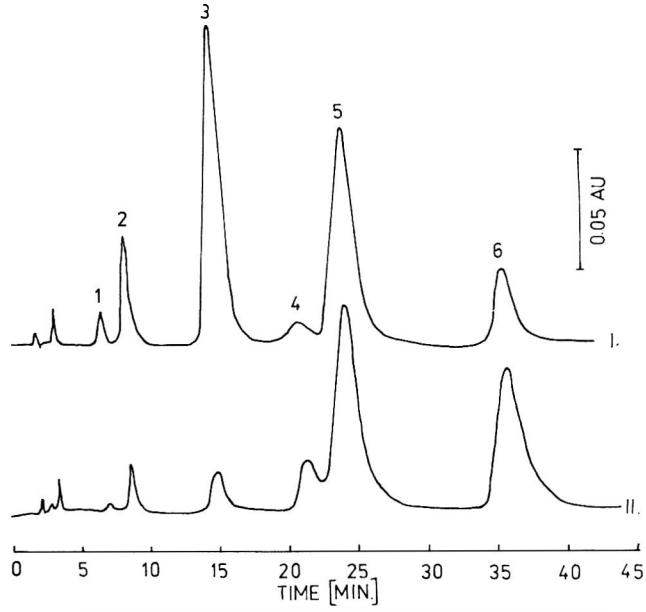

Fig. 11—Determination of Usal decomposition products formed in the buffer pH 2.9. I = after 224 days at 25°C; II = after 7.25 hr at 90°C. Peak identification: 1 = phenylalanyl-aspartic acid; 2 = phenylalanine; 3 = aspartyl-phenylalanine; 4 = phenylalanine methyl ester; 5 = diketopiperazine; 6 = Usal.

amino acids aspartic acid and phenylalanine or aspartic acid and phenylalanine methyl ester (E_4 and E_5). A comparison of E_2 (cyclization of aspartyl-phenylalanine to diketopiperazine), E_3 (diketopiperazine hydrolysis to aspartyl-phenylalanine) and E_7 (diketopiperazine hydrolysis to phenylalanyl-aspartic acid) shows that under the conditions applied (pH 2.9), the equilibrium is shifter towards the formation of diketopiperazine at higher temperatures.

The observed results are in accord with the proposed scheme of Usal decomposition (Fig. 1). The formation of phenylalanyl-aspartic acid was verified and its concentration in samples with Usal decomposed to 50% of the initial amount varied in the range of 0.1–1% at pH values 1.1–4.0 (Fig. 11 and 12). Similar dipeptide inversion (glycylisoleucine → diketopiperazine → isoleucylglycine) have been described by Steinberg and Bada (1981).

The concentrations of the resulting aspartyl-phenylalanine and diketopiperazine are strongly dependent on the pH value. With the higher pH value the content of diketopiperazine increased and the content of aspartyl-phenylalanine decreased (Fig. 2, 4, 13). Diketopiperazine concentration after the decomposition of 50% of Usal was from 3.5% at pH 1.1 to 25% at pH 3.9. In the pH range of 6.0–7.1 diketopiperazine was practically the only product formed (aspartyl-phenylalanine concentration was about 2%) (Fig. 4 and 13). The dipeptide aspartyl-phenylalanine was formed as the main decomposition product at lower pH values; at pH 1.1 it was the only decomposition product formed (Fig. 2 and 13). The formation of phenylalanine methyl ester was also dependent on the pH value. Its concentration at 50% Usal decomposition ranged up to 3%.

The effect of temperature on the formation of Usal decomposition products is obvious, especially in the formation of aspartyl-phenylalanine and diketopiperazine (Fig. 11). More diketopiperazine and less aspartyl-phenylalanine were formed with increasing temperatures. The same observation was made by Scherz et al. (1983). The content of phenylalanine methyl ester (6%) was twice that at 80°C and 90°C after the decomposition of 50% Usal, in comparison with its concentrations at 25°C and 40°C. The distribution of the other products re-

—Text continued on page 1415

Table 3—Rate constants in relation to temperature at pH 2.9

Temp. (°C)	Rate constants (ks^{-1})								
	k_1	k_2	k_3	k_4	k_5	k_6	k_7	k_8	k_9
25	4.7×10^{-5}	8.6×10^{-3}	8.3×10^{-4}	4.2×10^{-6}	6.1×10^{-6}	5.3×10^{-5}	9.4×10^{-6}	1.1×10^{-2}	2.3×10^{-6}
40	2.1×10^{-4}	3.6×10^{-3}	1.9×10^{-3}	2.4×10^{-5}	1.8×10^{-6}	8.6×10^{-5}	1.8×10^{-4}	8.9×10^{-3}	1.1×10^{-2}
60	1.3×10^{-3}	1.9×10^{-2}	4.2×10^{-3}	2.2×10^{-4}	4.2×10^{-4}	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-4}	6.9×10^{-4}
70	3.3×10^{-3}	6.7×10^{-2}	9.7×10^{-3}	7.8×10^{-4}	1.3×10^{-6}	2.1×10^{-3}	3.6×10^{-3}	3.6×10^{-6}	4.2×10^{-3}
80	9.7×10^{-3}	1.5×10^{-1}	1.8×10^{-2}	2.0×10^{-3}	9.7×10^{-3}	2.8×10^{-4}	3.6×10^{-2}	8.1×10^{-2}	1.2×10^{-3}
90	1.4×10^{-2}	4.4×10^{-1}	2.8×10^{-2}	4.2×10^{-3}	9.4×10^{-3}	1.6×10^{-2}	8.6×10^{-4}	4.7×10^{-5}	1.3×10^{-1}

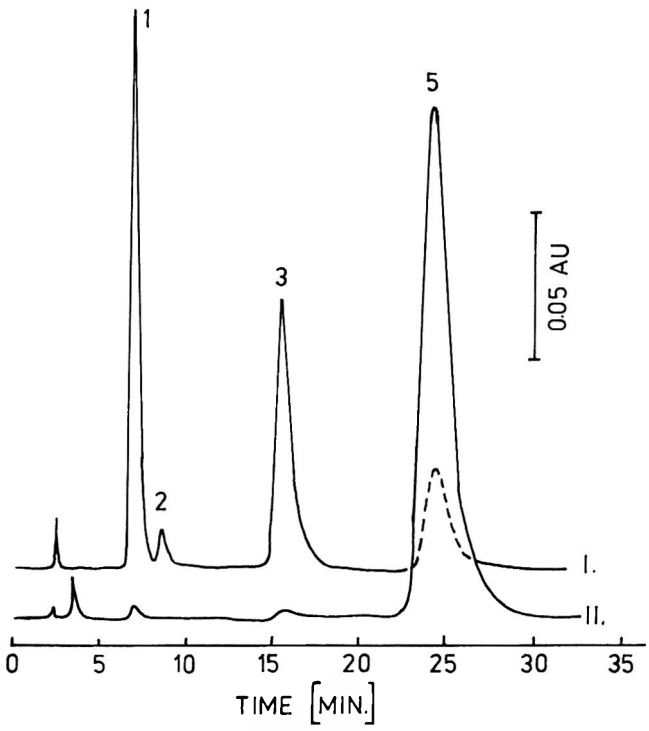

Fig. 12—Separation of diketopiperazine decomposition products formed after 10 days of storage at 25°C in buffer. I = pH 1.2; II = pH 3.3. The initial diketopiperazine concentration was 2 mg·mL⁻¹. Peak identification the same as in Fig. 11.

Table 4—Activation energies at pH 2.9

Activation energy (kJ/mole)						
E_1	E_2	E_3	E_4	E_5*	E_6*	E_7*
79	92	49	95	100	85	113

* Calculated from higher dispersion data

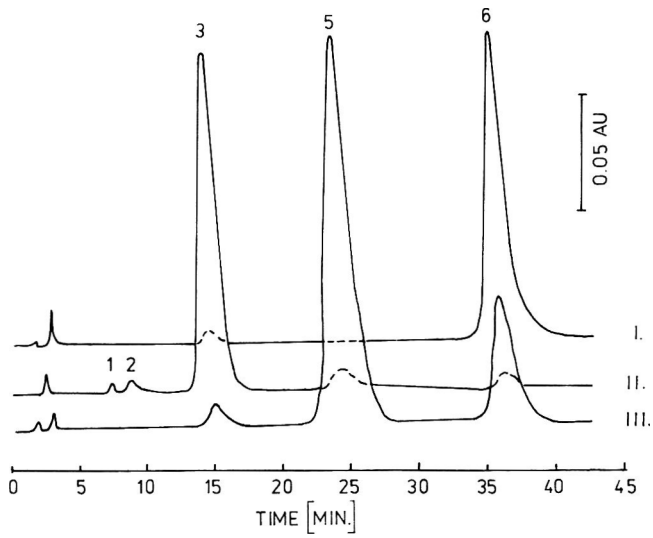

Fig. 13—Determination of Usal decomposition products in: I = fresh Usal solution (5 mg·mL⁻¹); II = buffer pH 1.1 after 31 days of storage at 25°C; III = buffer pH 7.1 after 23 hr of storage at 25°C. Peak identification the same as in Fig. 11.

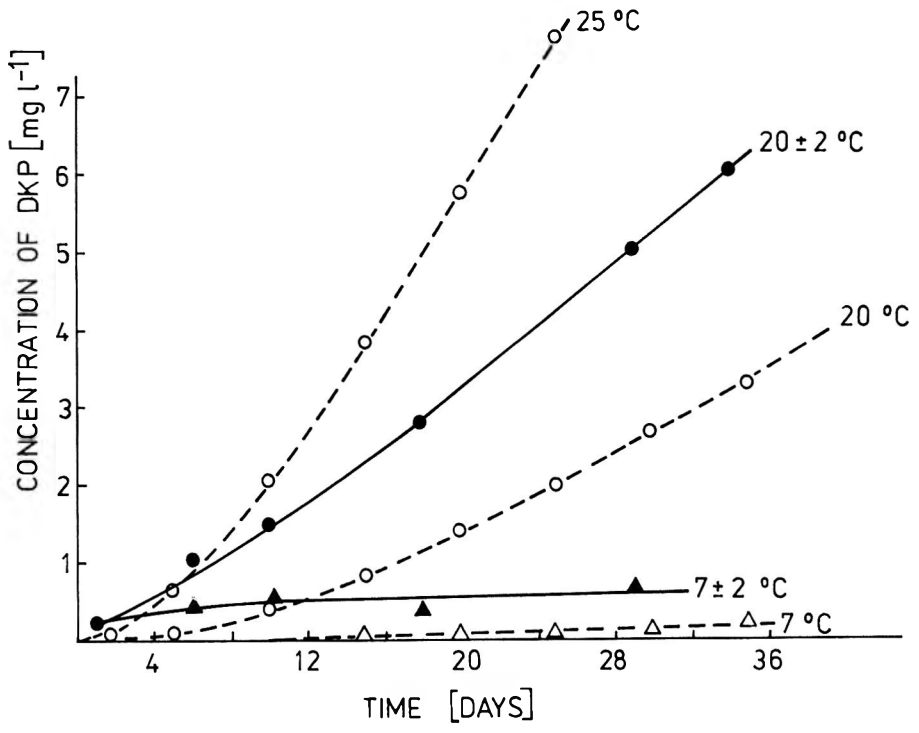

Fig. 14—Course of diketopiperazine (DKP) formation in the soft drinks DIA TONIC and DIA EXOTIC. △, ○ = calculated values of the theoretically resulting diketopiperazine for the given conditions (- - - - -); ▲, ● = experimental data obtained in the soft drinks (—).

Ultrastructure of Fresh, Airblast and Sodium Chloride Brine Frozen Uncooked Blue Crab Muscle

C. K. COLEMAN, F. K. AL-BAGDADI, S. L. BIEDE, and C. R. HACKNEY

ABSTRACT

The ultrastructural changes of 0°C blast-frozen and brine-immersed (23% NaCl at 0°C) blue crab (*Callinectes sapidus*) levator muscle were studied by transmission electron microscopy in comparison to unfrozen, *prerigor* muscle. Characteristic, striated skeletal muscle features were seen in fresh muscle, including double M-lines and Z-tubules seen in some Crustacea. Changes in structural organization caused by blast freezing (slow method) included decreased density and change in shape of myofibrils and Z-lines, and disappearance of the sacroplasmic matrix. Disintegration of Z-lines, myofibrils, and sarcoplasm was less severe due to brine-immersion freezing (rapid method). Distances between myofibrils were compacted in brine-immersed blue crab muscle samples, related to firmer objective texture measurements.

INTRODUCTION

IN FISHERY PRODUCTS, most of the electron microscopy studies of structural changes during freezing have been conducted on finfish (Bello et al., 1981, 1982; Jarenback and Liljemark, 1975 a, b; Liljemark, 1969; Tanaka, 1965; Partmann, 1963). Published reports on crustacean muscle, which differs biochemically and structurally from vertebrate muscle, have been limited to comparisons of species characteristics in fabricated shrimp products, and the gross morphology of thermally processed shrimp and blue crab meats (Soo et al., 1978; Giddings and Hill, 1976, 1978; Atwood, 1972). Extracellular ice formation in slow frozen blue crab tissue (*Callinectes sapidus*) was shown to yield the greatest alterations within muscle fibers. Deterioration of textural quality in muscle foods has been related to fine structure changes resulting from denaturation of myofibrillar proteins and density of Z-lines which occurs during postmortem glycolysis and *rigor mortis* (Dunajski, 1979; Bendall, 1973). In fish particularly, increased toughness occurs with compacting of myofilaments (Jarenback and Liljemark, 1975a; Tanaka, 1965), while tenderness occurs with loss of proteinaceous material (Love, 1968).

Most frozen blue crab products are precooked meats incorporated into specialty items. This masks the developed undesirable, tough texture. Rapid freezing methods investigated and scored highly by sensory panels have not been accepted commercially for precooked, picked meats (Ampola and Learson, 1971; Strassner et al., 1971). Along the Gulf coast of the United States a style of frozen blue crabs is packaged and marketed as uncooked body cores, termed "gumbo crabs." These processed crabs, blast frozen and held at −18°C, are used in soups by the consumer, with or without thawing. Comparison of frozen gumbo crabs with fresh blue crabs, when used in soups, have shown the frozen crabs to develop a soft, stringy, noncohesive texture upon cooking.

The objective of this study was to provide information on the ultrastructural changes occurring in frozen uncooked blue crab meat prior to cooking which may influence the quality of the cooked product.

Author Coleman is with the Dept. of Food Science, Lousiana Agricultural Experiment Station, Louisiana State Univ. Agricultural Center, Baton Rouge, LA 70803. Author Al-Bagdadi is with the Dept. of Veterinary Anatomy & Fine Structure, School of Veterinary Medicine, Lousiana State Univ., Baton Rouge, LA 70803. Author Biede is with Mead Johnson & Company, Food Product Development, Evansville, IN 47221. Author Hackney is with Virginia Polytechnic Institute & State Univ., Seafood Processing Research & Extension Unit, 102 S. King St., Hampton, VA 23669. Address inquiries to Dr. Hackney or Dr. Coleman.

MATERIALS & METHODS

CRABS employed in this study were harvested commercially in Louisiana and purchased from a local processor. Control samples, held live in a 4°C cold room, were euthanized with ether and sampled immediately after death. Live, whole crabs were frozen by (1) holding at 0°C for 3 hr (identified as "slow frozen") or (2) submersing in a 23% NaCl brine at 0°C for 15 min (identified as "rapid-frozen"). Subsequent to killing, the whole crabs were backed, eviscerated, and halved. Frozen halves were placed in 25 cm × 30 cm polyethylene pouches, heat sealed, stored at −20°C, and sampled after 135 days. After thawing in a 4°C cold room, muscle fibers were dissected directly from intact crab halves, after cutting away the thin endoskelton with scissors and forceps.

Sample preparation

Blocks of muscle tissue (1 cm^3) were obtained from the central *musculus levator* of the fifth leg (backfin) base musculature (Cochran, 1935). Transverse sections of individual fibers (0.5–1 mm^3) were chopped in a lake of fixative, then held in the same fixative at 4°C (2.5% glutaraldehyde in sodium phosphate buffer (66% v/v 0.2M $Na_2HPO_4 \cdot 7H_2O$, 0.2M $NaH_2PO_4 \cdot H_2O$, pH 7.1) for 90 min. Fibers were washed in the same buffer at 4°C, four 15 min changes, with periodic agitation. Post-fixation occurred in 1% osmium tetroxide/sodium phosphate buffer, pH 7.1, for 60 min at room temp. Samples were washed again in the buffer at 4°C (4 × 15 min changes, then overnight). Dehydration changes were two × 10 min, in 30, 60, 70, 80, and 90% ethanol, room temp (held overnight in 70%). Final dehydration occurred in absolute ethanol, three 20 min changes at room temperature. Propylene oxide, three 10 min changes, cleared the tissue of ethanol in preparation for embedding.

Epon 812 (Ernest F. Fullman, Inc.) stock mixtures were Epon A (5 mL Epon 812, 8 mL dodecenyl succinic anhydride) and Epon B (8 mL Epon 812, 7 mL nadic methyl anhydride). Tissue fibers were infiltrated using a ratio of propylene oxide/Epon 812 Embedding Medium (13 mL Epon A, 15 mL Epon B); 1:1, three 1 hr changes; 1:2, one 2 hr change; and 1:4, one change overnight (room temp). Polymerization occured in Epon 812 Embedding Medium (13 mL Epon A, 15 mL Epon B, 16 drops DMP-30 accelerator (2,4,6-tri-(dimethylamino-methyl)phenol, 60 hr at 45°C. Silver sections were prepared using an LKB ultramicrotome equipped with a diamond knife. Sections were mounted on uncoated copper grids (200-300 mesh) and stained with uranyl acetate, followed by Reynolds lead citrate (Reynolds, 1963). A Zeiss 10 electron microscope, 80 Kv, was used for observation.

RESULTS & DISCUSSION

THE ORGANIZATION of myofibrils in the skeletal muscle tissue of blue crab *levator* muscles was similar to that observed in other Crustacea and fish. The muscle fibers (Fig. 1 and 3) exhibited the characteristic parallel arrangement of skeletal

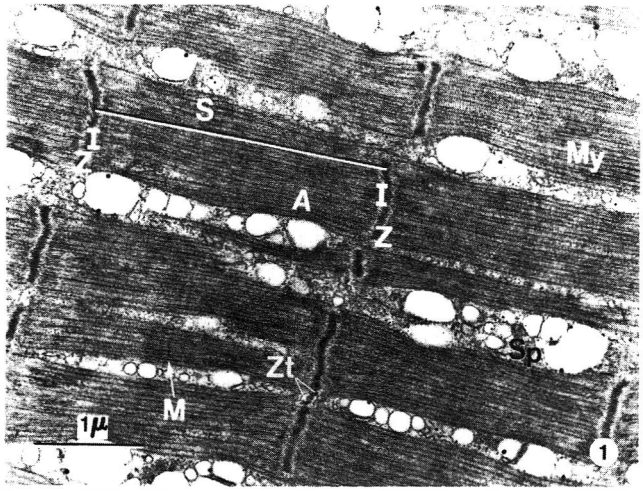

Fig. 1—Transmission electron micrograph of a longitudinal section of prerigor, unfrozen blue crab levator muscle containing sarcomeres (S), I-bands (I), A-bands (A), myofilaments (My), Z-lines (Z), double M-lines (M), sarcoplasma (Sp), and Z-tubules (Zt).

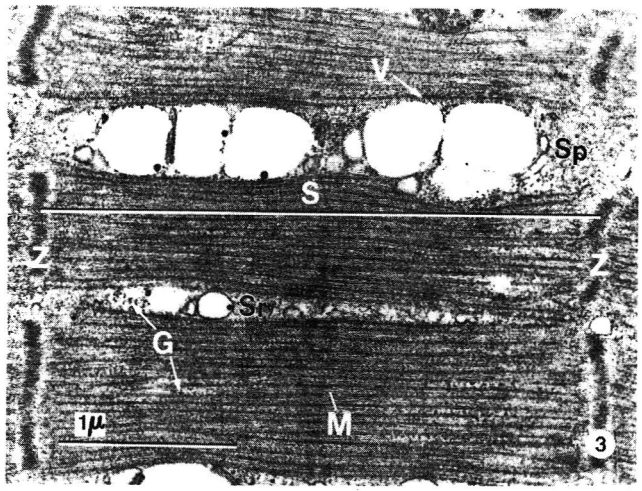

Fig. 3—Transmission electron micrograph of unfrozen levator muscle showing three sarcomeres (S) in register, Z-lines (Z), M-lines (M), glycogen granules (G), sarcoplasmic reticulum (Sr), and vesicles (V) within the sarcoplasm (Sp).

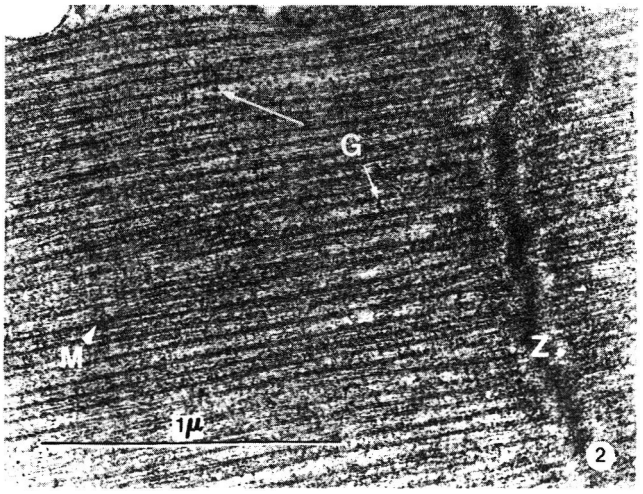

Fig. 2—Transmission of electron micrograph of unfrozen levator muscle at high magnification showing a Z-line (Z), double M-line (M), and glycogen granules (G).

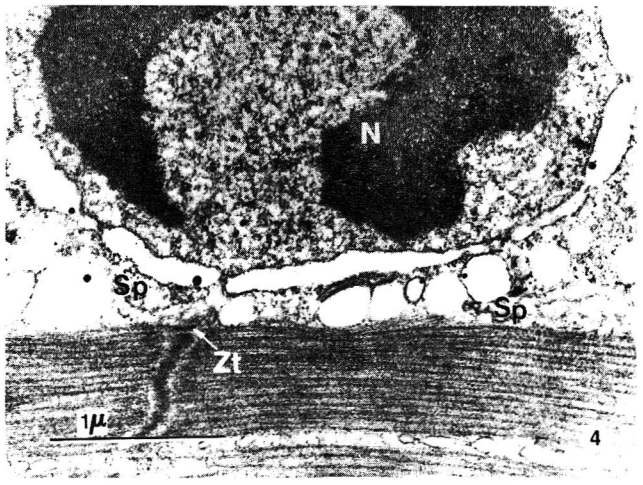

Fig. 4—Transmission electron micrograph of unfrozen levator muscle containing the sarcoplasm (Sp), a nucleolus (N), and a Z-tubule (Zt).

muscle fibers containing aligned contractile units, the sarcomeres (S). A prominent feature of this muscle was the irregularity of myofibril shape and width, even compared to the known variance of crustacean fibers (Chapple, 1982; Atwood, 1972; Franzini-Armstrong, 1970). Extremely narrow fibers were observed adjacent to wide fibers and sections of cojoined fibers (Fig. 1). The uniform, symmetrical myofibrillar patterns seen in fish and mammalian muscle tissue were not demonstrated in blue crab muscle. Dark Z-lines (Z) delineating the sarcomeres were well aligned, compared to most other types of Crustacea muscle. The Z-lines were dense, wide and slightly wavy (Fig. 1 and 2). The sarcomeres were further divided into the fundamental A-bands (A) and very narrow I-bands (I), and showed well distinguished thin and thick myofilaments (My) (Fig. 1). The thick filaments of the A-band extended almost to the Z-line. The diminuitive size of the I-band and the thin filaments not being seen autonomously indicated the fresh tissue myofibrils were in a contracted state. A darkened area, suggested to be double M-lines (M), was observed in the center of most A-bands of the unfrozen blue crab muscles. An H-zone could not be identified, which could be due to the muscular contraction (Fig. 1, 2, and 3). The H-zone is usually light in appearance in stretched muscle, due to the absence of thin, actin filaments within the pattern of interdigitating myofilaments. The shadowed central area most often appeared as two dense parallel lines, previously described as double M-lines in shrimp locomotory and crab swimming musculature (Hoyle, 1973; McNeill et al., 1972).

Between the myofibrils, the sarcoplasm (Sp) contained an assortment of large and small vesicles (V) (Fig. 3). An occasional sectioned nucleolus (N) was present (Fig. 4). Large and abundant vesicles of varied sizes were also exhibited in lobster tail and Alaska pollack muscle (Hayes et al., 1971; Tanaka, 1965). Here, (Fig. 1), the large relative vesicular size seen in *prerigor* blue crab muscle was greater in proportion to the myofibrillar width than that observed in other Crustacea and fish. Uniform diad patterns between the sacroplasmic reticulum (Sr), and elongated T-tubules, common in vertebrates, were not evident within these vesicles (Fig. 3). At intermittent locations, pairs of Z-tubules (Zt), (Fig. 1 and 4), seen in certain crab and crayfish muscle tissue, manifested characteristic electron dense material (Chapple, 1982; Hoyle, 1973; Morin and McLaughlin, 1973; Franzini-Armstrong, 1970; Peachey and Huxley, 1964). Glycogen granules (G) were observable within the sarcoplasma, as well as in the myofibrils (Fig. 2 and 3).

The widely spaced structure of the unfrozen blue crab lump meat muscle, with its high proportion of sarcoplasmic fluid,

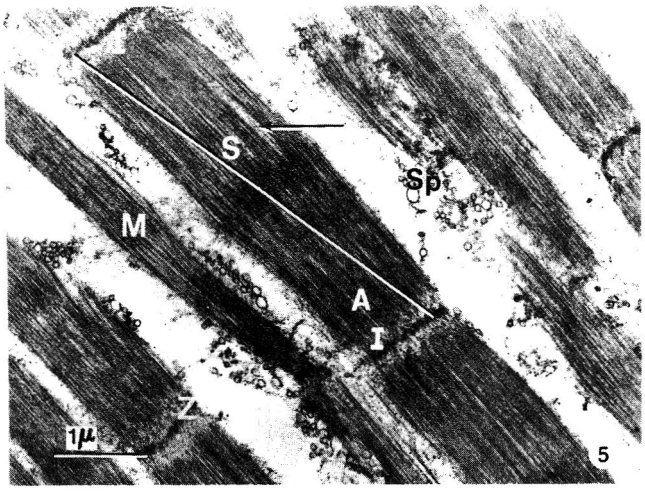

Fig. 5—Transmission electron micrograph of a longitudinal section of 0°C blast frozen (slow), 135 day storage, blue crab levator muscle containing sarcomeres (S), Z-lines (Z), A-bands (A), I-bands (I), M-lines (M), sarcoplasm (Sp), and gaps between myofilaments (arrow).

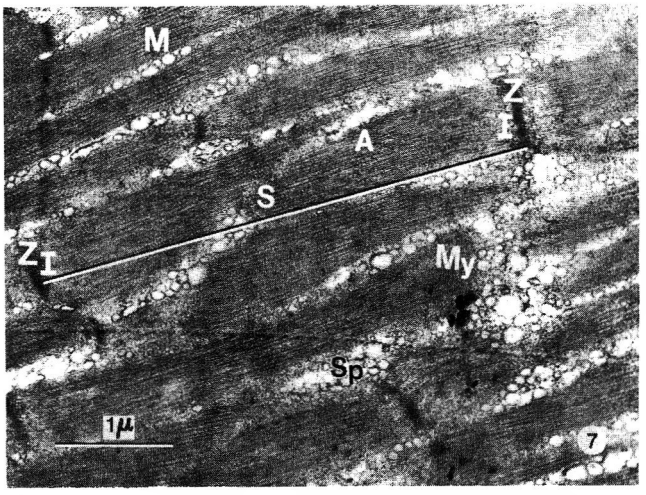

Fig. 7—Transmission electron micrograph of a longitudinal section of blue crab levator muscle immersed in salt brine at 0°C (rapid), 135 day storage. Sarcomeres (S), A-bands (A), I-bands (I), Z-lines (Z), myofilaments (My), M-lines (M), and the sarcoplasma (Sp) are shown.

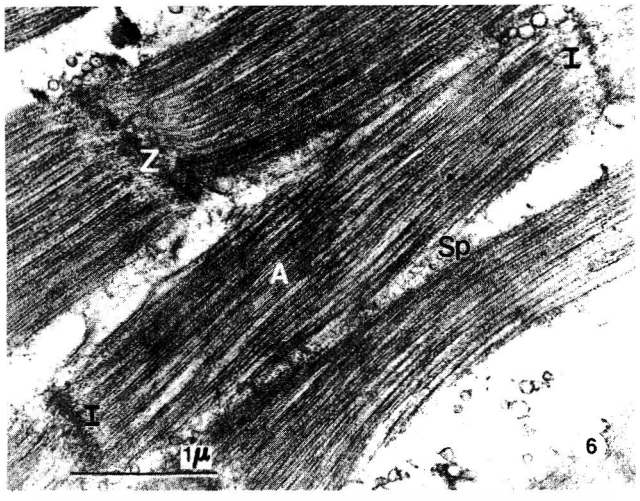

Fig. 6—Transmission electron micrograph of slow frozen, stored, levator muscle demonstrating Z-lines (Z), I-bands (I), A-bands (A) and sarcoplasm (Sp).

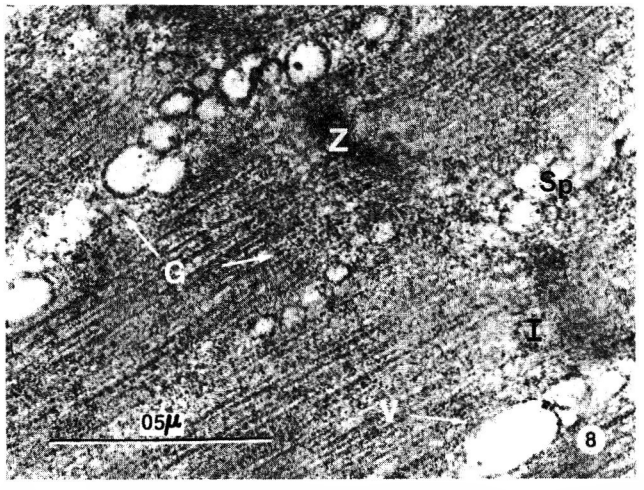

Fig. 8—Transmission electron micrograph of rapid frozen, stored, levator muscle with vesicles (V) within the sarcoplasm (Sp), Z-lines (Z), I-bands (I), and glycogen granules (G).

suggested a system which would allow fibrils to easily slide past one another upon compression. The high liquid content and sparse framework of muscle fibers would contribute to the characteristic soft texture of blue crab lump meat.

The combination of slow freezing (0°C) and 135 day storage at −20°C, illustrated in Fig. 5, 6, and 9, created distortion of the regular fine structure of the blue crab muscle. The sarcomeres (S) of the myofibrils remained intact in most cases, but the character of the intermyofibrillar space and Z-lines (Z) changed.

The slow freezing treatment caused a decrease in the density of the myofibrils (Fig. 5, 6, and 9). The thick and thin filaments constituting the sarcomeres were evident, but the spacing between them was wide, irregular, and showed gaps which might be created by ice crystal formation between the protein fibers (arrow) (Fig. 5). Light I-bands (I) clearly contrasted with the adjacent A-bands (A). The alignment of thin filaments in the I-bands was not parallel; the actin myofilaments appeared fragmented and indistinct in their attachment to the Z-line (Fig. 5 and 6).

A single, indefinite dark band (M) was observed in the center of each muscle sarcomere of the slow frozen method, in contrast to the double M-lines seen in unfrozen controls (compare Fig. 1 and 5). Often, the shape of each contractile unit within the myofibril swelled at this central band, presenting a bowed appearance, pinched in at the Z-lines. Other sarcomeres were fragmented, misshapen, and curled.

The densities of the Z-lines were disrupted in the slow frozen muscle tissue compared to unfrozen controls (Fig. 9). Gaps were seen within the Z-lines, and inherent structural proteins spread into less concentrated, diffuse configurations. Disruption of Z-lines has been associated with postmortem changes by proteolysis and weakening of proteins by pH changes. Freezing and low temperature storage of fish muscle has also been shown to cause deterioration of Z-lines (Dunajski, 1979; Bendall, 1973). Whole crabs preserved by slow blast-freezing would be susceptible to a period of *rigor* contracture, as well as additional damage by ice crystal migration during frozen storage. Disintegration of the structural framework of skeletal muscle tissue would decrease the resistance to compression during textural analysis, and render the meat soft and infirm.

The space normally occupied by components of the sarcoplasm (Sp) was distinctly enlarged and void of material in the slow frozen blue crab muscle tissue (Fig. 5 and 6). Compare the abundant material visible in unfrozen crab muscle (Fig. 1).

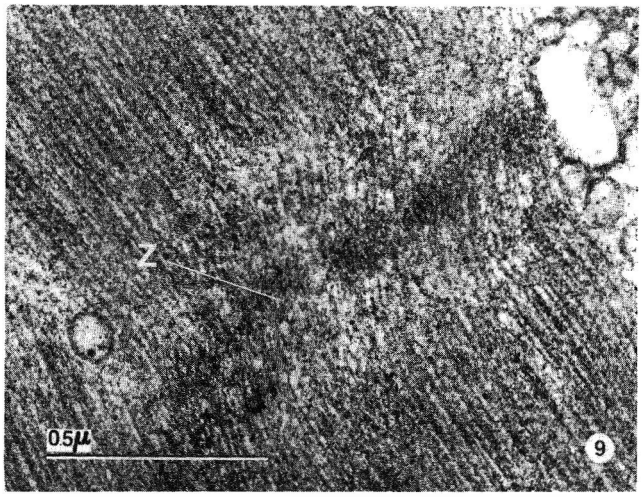

Fig. 9—Transmission electron micrograph of a Z-line (Z) of slow frozen, stored blue crab levator muscle at high magnification.

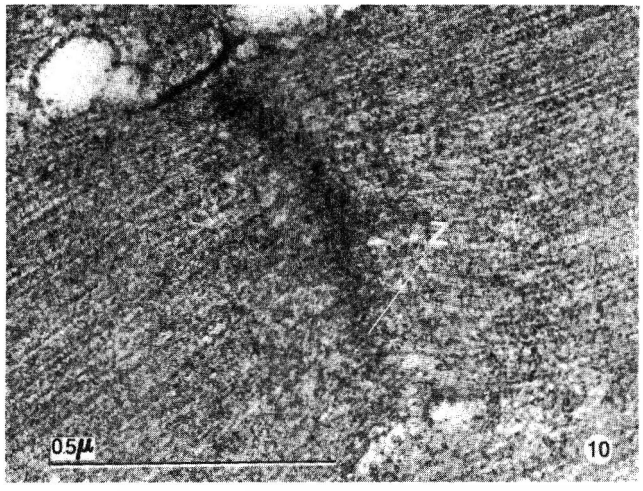

Fig. 10—Transmission electron micrograph of a Z-line (Z) of rapid frozen, stored blue crab levator muscle at high magnification.

Glycogen granules were not clearly seen. The growth of large, extracellular ice crystals during slow freezing and storage disintegrated the vesicles and matrix of the sarcoplasm, while displacing the myofibrils. Precipitates and small vesicles were scattered throughout the interfibrillar spaces. Loss of the fiber network and breakdown of the myofibrils and vesicles would weaken physical stability of the tissue and relate to textural tenderness.

The effects of rapid freezing (salt brine at 0°C) and storage at −20°C for 135 days on blue crab *levator* muscles have been depicted in Fig. 7, 8, and 10.

The myofibrils maintained a somewhat striated orientation after this treatment, with most fibers having straight, parallel sides (Figs. 7 and 8). Sarcomeres (S) appeared elongated due to the treatment, but contained constitutent Z-lines (Z), A-bands (A), and thick and thin myofilaments (My). The Z-lines were seldom in register, but did not present the pinching effect seen in slow frozen muscle tissue. The darkened lines central to the A-band in fresh and slow frozen samples, suggested to be M-lines (M), were obscure or absent in rapid frozen blue crab muscle (Fig. 7). Z-line disintegration was less severe than that seen in slow frozen specimens (Fig. 8 and 10). The lines broadened and decreased in density, but showed fewer interruptions of protein structure compared to slow frozen skeletal muscle. As in the other treatment, light I-bands (I) were seen (Fig. 7 and 8), as disorganized regions with indistinct thin filaments. The connection of thin filaments to the Z-line was not clearly visible.

The area between myofibrils (Sp) contained abundant, distinct vesicles (V) and granular matrix similar to unfrozen blue crab muscle (Fig. 7 and 8). However, the distance between myofibrils and constituent myofilaments decreased, rather than increased, by the degradation effect of rapid freezing and cold storage, similar to that reported by others in frozen fish tissue (compare Fig. 5 and 7). This compacting of fine structure myofibrils would correlate with increased firmness during texture measurements, even with some loss of the characteristic juiciness of fresh blue crab meat. Objective shear/compression measurements of whole lump portions of blue crab muscle, frozen by the same methods, demonstrated rapid frozen samples retained greater firmness compared to slow frozen crab portions. Glycogen (G) particles could still be seen in the sarcoplasm of 0°C salt brine frozen muscle tissue (Fig. 8).

Rapid frozen blue crab muscle maintained a loose structure of tissue components after storage compared to other marine species (Bello et al., 1982; Liljemark, 1969; Tanaka, 1965). This was related to the original, highly liquid nature of unfrozen blue crab muscle ultrastructure. High scores for tenderness and physical softness, rather than the progressive toughening of fish muscle, may be attributed to the loose fine structure demonstrated in these transmission electron micrographs. Gross morphology of the blue crab backfin lump muscle showed multi-bundled fiber clusters and very little connective tissue between fibers, contributing to the muscle unit's textural softness (Spirito, 1972).

In summary, superior preservation of the sarcoplasmic matrix and myofibrillar components by the rapid, brine-freezing method has been shown to be more advantageous in maintaining blue crab meat structural quality than slow, blast-freezing. In related studies, textural and chemical composition of frozen uncooked blue muscle are being compared to fully evaluate the benefits of the methods for industry.

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Chemical and Physical Changes in Red Hake Fillets During Frozen Storage

YAW J. OWUSU-ANSAH and HERBERT O. HULTIN

ABSTRACT

Changes in chemical and physical properties of frozen red hake fillets were determined as a function of time. The decrease in protein extractability could be partly reversed by mercaptoethanol. Sodium dodecylsulfate greatly reduced the loss in extractability. Polypeptides that would not enter 10% polyacrylamide gels after treatment with SDS and mercaptoethanol were formed during frozen storage. The rate of decrease of the myosin heavy chain followed first order kinetics with a rate constant of −0.054 per week at −7°C and closely paralleled the appearance of the "cross-linked" peptides. Uniaxial compression moduli correlated well with the dimethylamine produced and the loss of the myosin heavy chain after SDS-PAGE.

INTRODUCTION

PROTEIN DETERIORATION is a major problem in frozen storage of fish and has, therefore, been the subject of much research and numerous review articles (Connell, 1964; Sikorski et al., 1976; Matsumoto, 1979; Shenouda, 1980). Gadoid fish contain an enzyme system which breaks down trimethylamine oxide (TMAO) to dimethylamine (DMA) and formaldehyde (HCHO) (Amano and Yamada, 1964; Yamada et al., 1969; Dingle et al., 1977; Crawford et al., 1979). The latter has been suggested to cause protein cross-linking resulting in textural deterioration of the fish muscle. Although there is no direct evidence that cross-linking of proteins in fish muscle is caused by endogenously produced HCHO, it has been shown that the enzymic demethylation product of TMAO, DMA, is a good estimator of textural deterioration in red hake (Gill et al., 1979). Using an Instron Universal Testing Machine, they measured both the slopes and the peak heights of the force deformation curves and found significant correlations for both parameters with sensory data; slopes appeared to provide a better indication of sensory toughness than did the peak heights. Johnson et al. (1980) converted the direct measurements of the Instron Universal Testing Machine to a true stress-strain relationship, a quantity termed the compressive deformability modulus. This procedure measures a more fundamental physical property than most physical measurements used to evaluate fish flesh, and the authors found it to be a sensitive index to textural properties.

The objective of this work was (1) to study the relation of the enzymic demethylation products of TMAO to the solubility properties of fish muscle proteins using an agent to disrupt disulfide bonds and a detergent which breaks up several types of protein interactions and (2) to compare the chemical data with the compressive deformability modulus of the raw flesh.

MATERIALS & METHODS

RED HAKE (*Urophycis chuss*), a gadoid fish, was chosen because of its known susceptibility to both TMAO breakdown and toughening during frozen storage. The fish were obtained from day boats in Gloucester and transported to the laboratory on ice. The fish were filleted and skinned immediately. Fillets of the same fish were separately packaged in polyethylene bags and labelled. Each of the paired fillets were fast-frozen at −80°C for 4 hr, then assigned to a storage temperature. The control fillets were stored at −80°C and the test fillets at −7°C for 16 wk; samples were removed for analyses every 2 wk.

Author Hultin is with the Massachusetts Agricultural Experiment Station, Dept. of Food Science & Nutrition, Marine Foods Laboratory, Univ. of Massachusetts Marine Station, Gloucester, MA 01930. Author Owusu-Ansah, formerly with the Univ. of Massachusetts Marine Station is now with POS Corporation, 118 Veterinary Road, Saskatoon, Saskatchewan S7N 2R4 Canada.

Measurement of physical properties

Compressive deformation moduli of the fillets were determined using an Instron Universal Testing Machine according to the procedure of Johnson et al. (1980). The dimensions of the specimens were 3–5 cm × 3 cm and the thickness ranged from 0.6–2.6 cm. The dimensions of each specimen were measured and similar specimens were prepared for both the control and test samples. The force-deformation data were converted to their stress-strain relationship and corrected for dimensional effects by the modified method (Johnson et al., 1980) of Lindley (1978). The results were reported as the mean of the difference between the moduli of the test samples and the controls. Each mean data point was calculated from twelve replicates, four determinations on each of three fish.

Chemical analyses

The muscle used for the physical measurements was minced in a Rival meat grinder, and a trichloroacetic acid (TCA) extract was prepared by homogenizing 25g duplicate samples for 1 min in a Waring Blendor with 25 mL deionized distilled water and 50 mL 10% TCA solution. The extract was suction filtered through a Whatman No. 1 filter paper and aliquots were taken for analyses of TMAO, DMA and HCHO.

TMAO in the TCA extract was reduced by the procedure of Yamagata et al. (1969) using conditions described by Parkin and Hultin (1982). The trimethylamine (TMA) produced was analyzed by the procedure of Dyer (1945) using 45% KOH in place of 50% K_2CO_3 as suggested by Shewan et al. (1971). TMAO content was calculated as the difference between TMA content after reduction and TMA content before reduction.

Dimethylamine (DMA) in the TCA extract was determined by the modified (Dyer and Mounsey, 1945) copper dimethyldithiocarbamate colorimetric procedure of Dowden (1930).

Free formaldehyde (HCHO) in the TCA extract was determined by the procedure described by Castell and Smith (1973) using the Nash reagent (Nash, 1953). Bound formaldehyde was defined as the difference between the DMA content (which is produced in equimolar amounts with formaldehyde from the enzymic breakdown of TMAO) and the assayable formaldehyde content.

Extractable protein (EP) was determined by extracting duplicate 6g minced samples with 120 mL chilled 0.02M $NaHCO_3$ solution at pH 7.2 containing 5% NaCl (Dyer et al., 1950) in a semi-micro Waring Blendor at low speed for 5 min. Protein content of the extract was determined by the biuret procedure of Gornall et al. (1949). Differential extractable protein was determined by treating duplicate 3g minced muscle samples with 60 mL of solutions of 4% sodium dodecyl sulfate (SDS) with and without 0.5% β-mercaptoethanol and with ice cold 0.02M $NaHCO_3$ solution (pH 7.2) containing 5% NaCl with and without 0.5% β-mercaptoethanol under the conditions stated above. The protein content of the extract was determined by the modified biuret procedure of Pelley et al. (1978).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples for electrophoresis were prepared by sonicating 3g minced muscle in 60 mL 4% SDS with 0.5% β-mercaptoethanol for 30 min.

Table 1—Dimethylamine (DMA), formaldehyde (HCHO) and extractable protein (EP) content of red hake fillets stored at −7°C

Wk of storage	DMA*	Free HCHO*	Bound HCHO	%EP*
2	[a]0.19	[a]0.06	[a]0.13	[a]79.2
4	[b]0.10	[a]0.08	[b]0.02	[a]72.0
6	[c]0.57	[b]0.15	[c]0.42	[b]59.4
8	[d]0.39	[c]0.10	[d]0.29	[c]38.3
10	[e]0.48	[c]0.11	[c]0.37	[d]47.8
12	[d]0.34	[c]0.10	[e]0.24	[d]44.3
14	[f]0.84	[b]0.16	[f]0.68	[a]15.9
16	[g]0.69	[b]0.13	[g]0.56	[e]21.8

* %EP data expressed as % relative to control (paired fillet) stored at −80°C. The control is set at 100% (absolute value of 5.9 ± 0.2 g protein/100 g fish). DMA and HCHO data are differences of mean values in mmoles per 100g sample for samples stored at −7°C. and corresponding control fillets stored at −80°C.

[a-g] Values in the same column with different superscripts are significantly different (n=4; P<0.05).

*Table 2—Compressive deformation moduli and mean force of deformation for red hake fillets stored at −7°C**

Wk of storage	Mean force of deformation (N)	Mean apparent deformation moduli (N cm^{-2}) Ea	Corrected deformation moduli N cm^{-2} k=0.5	k=0.7	k=0.9
2	[b]70.6	[a]0.89	[a]0.68	[a]0.59	[a]0.49
4	[c]86.0	[b]1.08	[a]0.69	[b]0.69	[b]0.59
6	[c]99.8	[d]2.54	[b]1.27	[c]0.98	[c]0.88
8	[a]49.8	[e]3.13	[c]1.77	[d]1.76	[d]1.37
10	[d]121.1	[b]1.08	[a]0.79	[b]0.69	[b]0.59
12	[d]131.4	[c]1.37	[b]1.07	[c]0.98	[c]0.88
14	[f]558.1	[g]11.57	[e]6.08	[f]5.29	[f]4.41
16	[e]517.5	[f]10.29	[d]4.80	[e]3.62	[e]3.31

* The data are the difference of stored fillets (−7°C) and corresponding paired fillets (control −80°C).

[a-g] Values in same column with different superscripts are significantly different (n=12; P<0.05).

An ultrasonic disintegrator (Model S-75, Branson Instruments, Inc.) with a 1 cm diameter tip was used and the temperature of the sample during sonication was maintained below 35°C. Complete solubilization of the samples required sonication. The solubilized samples were dialyzed against 25 mM Tris/HCl buffer (pH 7.4) containing 0.1% β-mercaptoethanol and 0.2% SDS. The ratio of the homogenate to the dialyzing buffer was 1:50. Dialysis was for about 4 hr after which the solution was changed and dialysis was continued for another 12 hr. Glycerol and bromophenol blue were added to the dialyzed samples before electrophoresis to a final concentration of 20% and 0.001% respectively.

A modification of the method of Porzio and Pearson (1977) was used for SDS-PAGE with 10% polyacrylamide gels. The modification involved introducing polyacrylamide into the formulation to a final concentration of 0.005% to give mechanical strength to the gel and to prevent gel elongation during destaining. The gels were fixed for 1 hr in 50% methanol and 15% TCA, stained with 0.1% Coomassie blue R-250 in 50% methanol and 7% acetic acid for 1.5 hr at ambient temperature, and destained in 25% isopropanol and 10% acetic acid with several changes until the background was clear. The destained gels were scanned on an Isco gel scanner (Model 1310) connected to an Isco AU-5 absorbance/fluorescence monitor and an Isco type 6 dual optical unit with a 580 nm filter. Both the scanning and chart speeds were 30 cm/hr. The myosin heavy chain was identified both by its mass and its position compared to standard patterns from muscles of many species.

Statistical Analyses

Linear regression analysis was used in all correlation analyses. Correlation coefficients were tested for significance using the t-test at 95% confidence level (Steel and Torrie, 1960). Comparison of means were made using Duncan's multiple range test at 95% confidence level (Steel and Torrie, 1960).

RESULTS

THE DIFFERENCES between DMA, HCHO and extractable protein (EP) contents of the paired red hake fillets as a function of storage time at −7°C and −80°C are presented in Table 1. The values for DMA, HCHO, and per cent extractable proteins varied from fillet to fillet despite the fact that the data were corrected by subtracting the control results from the corresponding test data using paired fillets. Since there is little change in these parameters in samples of red hake stored at −80°C (Kelleher et al., 1982), it is likely that this is a reflection of fish-to-fish variability. Assuming an equimolar production of DMA and HCHO, about 70% or more of the HCHO produced was bound, except for the fourth week.

The extractable proteins decreased generally with storage time although there was some variation. The data for per cent extractable proteins were significantly correlated with DMA and bound and free HCHO contents of the fillets ($P \leq 0.05$). The correlation coefficients were −0.89, −0.86 and −0.85, respectively.

The electrophoretic patterns of proteins from whole muscle stored at −7°C over a period of time are shown in Fig 1. The myosin heavy chain was the major protein band that decreased with increasing storage time. Along with the decrease of the myosin band was a concomitant appearance of protein that did not penetrate into the gels. The peak area ratios of the myosin chain band for the test fillets compared to the control fillets as a function of storage time is shown in Fig. 2. The decrease of the peak area ratio was initially slow, increased rapidly and then slowed again. A semi-log plot of the data gave roughly a straight line with a correlation coefficient of −0.966 ($P < 0.01$) and a rate constant of −0.054 per week. In addition to the myosin heavy chain, the bands for troponin T and myosin light chain 1 also decreased. Gill et al. (1979) also found a loss of troponin and myosin light chains by SDS-PAGE from myofibrils isolated from stored red hake muscle.

The mean force of deformation of the fillets increased with increasing storage time, except for the samples stored for 8 wk (Table 2). The mean force of deformation data were converted to their strain and stress ratios to give the apparent deformation moduli independent of the cross-sectional area of the specimen. The apparent deformation moduli increased steadily for the first 8 wk of storage, and declined for the tenth and twelfth weeks before they increased sharply after 14 and 16 wk of storage (Table 2).

Due to the flat shape of the samples the apparent moduli depended on both the tissue properties and the shape of the specimen. To resolve these confounding effects and be able to detect only the changes in tissue properties in relation to storage time, the apparent moduli data were corrected for their shape factor at various degrees of hardness (k) (Johnson et al., 1980). The results are shown in Table 2 where values of k of 0.5, 0.7 and 0.9 were used. The degree of hardness of red hake muscle is not known. At the lowest practical hardness factor level (k=0.5), there was no significant change in the physical properties of the fillets for the first 4 wk of storage after which the moduli significantly increased. At the two higher hardness factor levels, the textural changes in the fillets during the second and fourth weeks of storage were significantly different ($P \leq 0.05$). At all hardness levels the corrected deformation moduli were reduced in the samples stored for 10 and 12 weeks before they again increased.

Table 3 shows the correlation coefficients between the physical measurements, the chemical indices and the changes in the peak area ratios of the myosin heavy chain. The instrumental data were positively and significantly correlated with the DMA, HCHO and bound HCHO contents produced in the fillets. When the mean force of deformation was used as the physical deterioration index, significant positive correlation coefficients of 0.82 and 0.83 were obtained with DMA and bound HCHO, respectively. Significant negative correlations were observed between the mean forces of deformation and per cent extractable proteins or peak area ratios of the myosin heavy chain. When either the corrected or uncorrected forms of the physical indices were used, the decrease in the peak area ratios of the myosin heavy chain had a correlation coef-

*Table 3—Correlation coefficients of the chemical and instrumental data and the peak area ratio of myosin heavy chain band**

	Mean force (N)	E_a (N cm^{-2})	Corrected E_a (N cm^{-2}) k = 0.5	k = 0.7	k = 0.9	Peak area ratio of myosin heavy chain band
DMA	0.82	0.84	0.85	0.84	0.84	−0.83
Free HCHO	0.66	0.67	0.69	0.69	0.69	−0.75
Bound HCHO	0.83	0.85	0.86	0.85	0.86	−0.83
% extractable protein	−0.79	−0.81	−0.83	−0.84	−0.84	−0.94
Peak area ratio of myosin heavy chain band	−0.69	−0.69	−0.70	−0.70	−0.70	–

* Correlation coefficients in each row and column are significantly different at $P<0.05$.

Table 4—Per cent extractable protein in different solutions for red hake fillets stored at −7°C relative to corresponding fillets stored at −80°C.

Solutions wk of storage	5% NaCl in 0.02M $NaHCO_3$ pH 7.2	5% NaCl in 0.02M $NaHCO_3$ pH 7.2 + 0.5% β-mercapto-ethanol	4% SDS	4% SDS + 0.5% β-mercaptoethanol
0	*(5.9)	*(6.3)	*(11.9)	*(15.2)
2	a,f78.9	a,g89.6	a,g93.0	a,g92.2
4	a,f71.2	a,g85.2	b,g85.9	b,g86.8
6	b,f56.5	b,f58.0	b,g82.9	c,g80.5
8	d,f37.2	c,f40.4	a,g94.4	a,g92.2
10	c,f47.2	b,f58.2	b,c,h77.1	c,h80.6
12	c,f44.3	c,f47.9	c,g73.1	c,h81.2
14	e,f17.3	d,g27.4	c,h68.9	d,h74.2
16	e,f22.2	d,g29.2	c,h71.2	c,i80.8

* (g protein/100g muscle extracted from control and set at 100%).

a-e Values in the same column with different superscripts are significantly different ($n=4$; $P<0.05$).

f-i Values in the same row with different superscripts are significantly different ($n=4$; $P<0.05$).

ficient of −0.70. The correlations between the physical data, the chemical data and the peak area ratios did not change significantly ($P \geq 0.05$) when the physical measurements were corrected for their shape and hardness factors.

To identify some of the bonds involved in protein insolubilization during frozen storage, the extractability of the fillet proteins in various reagents was studied (Table 4). Per cent extractability in all solutions generally decreased with increasing storage time. For the first 4 wk of storage the per cent extractable proteins in the buffered 5% NaCl with β-mercaptoethanol was significantly higher than that extracted without β-mercaptoethanol. The enhanced extractability in the buffered salt solution containing the disulfide reducing agent was not significant from the sixth through the twelfth week of storage but was significant ($P < 0.05$) in the fourteenth and sixteenth weeks of storage. The increase in absolute amount of protein extracted (g protein/100g muscle) with mercaptoethanol in the salt solution was relatively small. Scans of the electrophoretic patterns of the extracted proteins during the fourth week of storage showed an increase in the peak areas of the myosin heavy chain and the actin bands by 9% and 13% respectively when β-mercaptoethanol was present in the extracting salt buffer. In the 14- and 16-wk stored samples, addition of β-mercaptoethanol to the extracting solution increased the extractability of actin (11% increase in electrophoretic peak area) but not that of the myosin heavy chain.

A 4% SDS solution markedly increased the protein extractability of the samples at all time periods. For the first 8 wk there was only a minimal loss of extractability in 4% SDS; between 8 and 10 weeks there was a significant loss in extractability in the detergent, but the loss in extractability did not increase over the 10- to 16-wk period. The addition of β-mercaptoethanol led to a further increase in protein extractability (up to 15.2g/100g of muscle tissue). Although on a percentage basis, β-mercaptoethanol only enhanced protein extractability in the presence of 4% SDS at the 12- and 16-wk storage periods, on an absolute basis the β-mercaptoethanol enhanced protein extractability at all time periods (note that the extractability of the 0 time sample with SDS plus mercaptoethanol was considerably greater than that of the sample with only SDS).

DISCUSSION

This study has reconfirmed previous ones with red hake which showed a general increase of DMA with time of storage of the frozen fish with a concomitant decrease in extractable protein (Dingle et al., 1977; Gill et al., 1979; Kelleher et al., 1982). The technique of using paired fillets in which the experimental fillet is compared with the fillet from the other side of the fish which has been stored at −80°C allows a direct determination of the experimental variable between comparable samples. A possible disadvantage is that it may emphasize fish-to-fish variability. This is perhaps one of the reasons why the values for the chemical determinations do not increase (or decrease) in a consistent pattern. There was a close relationship between the increase in DMA and the decrease in EP in that when there was a relatively high value of DMA, there usually was a relatively low value of per cent EP. Thus, the correlations that have been observed with DMA and extractable protein hold not only as a function of time, but also with individual fish.

As has been shown with minced red hake (Banda and Hultin, 1983; Owusu-Ansah and Hultin, 1987) the majority of formaldehyde that is produced is not detectable by the assay employed. We believe that this is due to the interaction of the formaldehyde with constituents of the muscle thus preventing detection of HCHO. Where minced tissue has been separated into insoluble high molecular weight compounds, soluble high molecular weight compounds, and low molecular weight soluble compounds, it has been demonstrated that a large proportion of the bound formaldehyde interacts with the small molecular weight compounds (Owusu-Ansah and Hultin, unpublished). This work was based on a separation of the various fractions of the minced muscle prior to storage in the frozen state and generation of formaldehyde. Since it was not possible to fractionate the fillets in this manner prior to frozen storage, no information is available as to whether a major portion of the nonreactive formaldehyde in fillets likewise interacts with the small molecular weight fraction of the muscle.

The use of 4% sodium dodecylsulfate (SDS) as the extraction medium greatly increased the amount of protein extracted compared to the salt solution. There was some decrease in the extractable protein in SDS with storage time, but it was much less than the decrease that was observed in the presence of the high salt solution. This implies that hydrophobic interactions are at least partially involved in the loss of protein extractability with time of storage.

The disulfide splitting compound β-mercaptoethanol had a greater effect when the primary extractant was SDS than when it was the high salt solution as indicated by the increase in absolute quantity of EP at 0 time. In fact, the total extractable protein (15.2g/100g of muscle) that was obtained in the 0 time sample in the presence of 4% SDS and 0.5% β-mercaptoethanol was close to the total amount of protein in the sample. Red hake contains approximately 16.5g protein/100g of muscle (Kelleher et al., 1982). On a percentage basis, there was not a great deal of difference between the protein extractability

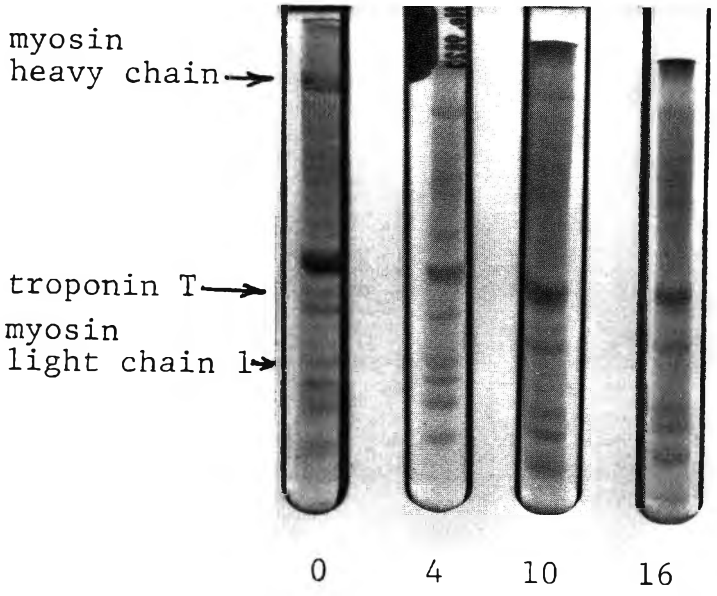

Fig. 1—Gel electropherograms of red hake fillet proteins on 10% SDS-polyacrylamide gels. Each gel had 100μg proteins. The number under each tube indicates the weeks of storage at −7°C.

with the 4% SDS solution and the same solution with the added sulfhydryl reagent. However, it must be kept in mind that the initial level of protein extraction was much higher in the latter, and it maintained this higher extractability on an absolute basis throughout the period of storage.

The greater effect of the sulfhydryl reagent on protein extractability in the SDS solution than in the high salt solution can be interpreted to mean that there are some disulfide bonds formed which are not exposed to the β-mercaptoethanol in the presence of high salt. However, when the detergent SDS was used, either the proteins were disaggregated or individual proteins were unfolded such that the β-mercaptoethanol had access to the disulfide bonds. These latter could then be broken to an extent such that there was a marked increase in protein extractability.

Matthews et al. (1979) in studying frozen stored minced cod observed an initial decrease in the amount of myosin heavy chain and the concomitant appearance of a band of material which did not enter 8.75% polyacrylamide gels. The amount of protein which did not enter the gel initially could be reduced in the presence of β-mercaptoethanol. However, on longer term storage, a high molecular weight band appeared that was not susceptible to the β-mercaptoethanol. Reaction of formaldehyde with myosin heavy chain was suggested as the cause of this high molecular weight fraction. Similar high molecular weight proteins were observed in frozen minced Greenland halibut (Lim and Haard, 1984). Aggregates of approximately 500,000 and in excess of 1,000,000 daltons were still intact after treatment with SDS and β-mercaptoethanol. Greenland halibut is a species which is not thought to produce significant amounts of formaldehyde by the breakdown of TMAO since it is not a gadoid. In our study, in a semi-quantitative way, it could be seen that there was an increased amount of protein which did not enter the SDS-PAGE gels with time of frozen storage. The primary loss was the myosin heavy chain, and this was concomitant with the increase of the high molecular weight fraction not entering the gel. There was also a loss of troponin T and myosin light chain 1. Although our results were

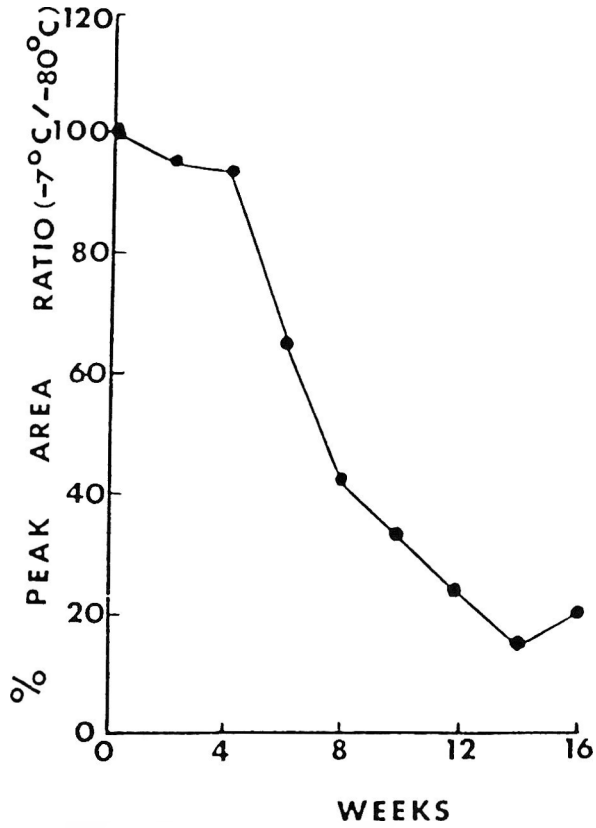

Fig. 2—Changes in the myosin heavy chain band peak area ratio as a function of storage time.

similar to that of Matthews et al. (1979), they were contrary to those of Gill et al. (1979) who found most of the changes occurring in the low molecular weight proteins. This discrepancy may be accounted for by the difference in sample preparation techniques. Gill et al. (1979) determined their electrophoretic patterns on isolated myofibrils. Thus, their extraction procedure was selective. Childs (1973) demonstrated that although the amount of protein that was extractable from fish muscle decreased when formaldehyde was added to the extracting buffer, myofibrils that were extractable had complete protein complements. This could be interpreted to mean that treatment of muscle, at least with exogenous formadehyde, leads to the development of more than one class of myofibrils,

at least one of which is modified by the exogenous formaldehyde.

We do not have a good quantitative measure of the amount of high molecular weight proteins that did not enter the gels. However, about 20% of the total proteins of the stored fish was insoluble without sonication in the 4% SDS and 0.5% β-mercaptoethanol extracting solution after storage for 16 wk. Since the high molecular weight fractions persisted even in these solutions, it might be supposed that covalent bonds are involved. These bonds were possibly formed by reaction with the formaldehyde produced. It should be emphasized, however, that the SDS-PAGE system will observe only cross-linking of peptides when they become so large that they do not enter the gel, i.e., greater than the 200,000 dalton component of myosin. It is possible that other cross-linking takes place between smaller peptides which is not discerned by this technique.

The measurement of the physical properties of fish flesh is difficult and the problems have been discussed (Johnson et al. 1980). We used the compressive deformability modulus of these authors to measure the physical properties of the raw red hake fillets as a function of time. This procedure converts the direct measurements of the Instron Universal Testing Machine to a true stress:strain relation. Significant correlations were obtained between the physical measurements and chemical determinants of deterioration. Correlations between mean force of deformation or the apparent deformation moduli with either DMA or bound formaldehyde were high, and there were significant negative correlations between the physical measurements and the per cent extractable protein. There was also significant correlation between the peak area ratios of the myosin heavy chain band and the physical measurements. Our data indicated that there was no obvious improvement in the interpretation of the results by correcting the data for cross sectional area, shape or hardness factors when paired fillets were used. This is consistent with the observation of Gill et al. (1979) who used data taken from force deformation curves and found significant correlations between chemical and physical measurements similar to those we observed. Thus, the simpler approach used by the latter workers is sufficient.

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Thermal Activation of Actomyosin Mg^{2+}-ATPases from Flying Fish and Black Marlin Muscles

TAKESHI TAGUCHI, MUNEHIKO TANAKA, YUJI NAGASHIMA, and KEISHI AMANO

ABSTRACT

The effects of heat treatment (25°, 30°, 35°, 37°, and 40°C) on actomyosin Mg^{2+}-ATPase activity from flying fish and black marlin were studied with respect to setting of their meat pastes. The setting ability of flying fish had very high values but black marlin was low. Flying fish actomyosin Mg^{2+}ATPase was activated more than three times by heating at 35°C, while the activation of black marlin was about two-thirds at 37°C. For acto-HMM ATPase, however, no appreciable difference in activation was observed between flying fish and black marlin. For both fishes, the acto-S-1 ATPase activities were not enhanced by the heat treatment. A possible role of myosin fragments in this activation is discussed.

INTRODUCTION

SOME FISH ACTOMYOSIN Mg^{2+}-ATPases are dramatically activated by thermal treatment at a given temperature (Taguchi et al., 1978). The level of thermal activation varied widely among fish species. The decreasing order of activation level is as follows: Alaska pollack, sardine, white croaker, horse mackerel, mackerel, carp, crucian carp, marlin groups. The order exhibited a tendency corresponding to the gel-forming abilities in "setting" of fish meat paste. The setting process is very useful for kamaboko manufacture in Japan.

Studies of the thermal gelation of muscle proteins have been reported by many workers (Yasui et al., 1980, 1982; Samejima et al., 1981; Lanier et al., 1981, 1982; Niwa et al., 1982; Acton et al., 1981; Shimizu et al., 1981, 1983; Montejano et al., 1983, 1984; Wu et al., 1985a,b). However, the exact characteristic of fish muscle protein possessing different ability to "set" is not yet fully understood.

The possible factors affecting the difference in gelation of muscle protein among fish species are as follows: (1) defference in development of hydrophobic interaction in the surface of protein molecules (Niwa et al., 1981a,b; Liu et al., 1982), (2) difference in thermal stability of myosin (Shimizu et al., 1983), (3) on the basis of protein-protein interaction (Acton et al., 1981), difference in dependence on the ability to interact between myosin and actin, or myosins.

To investigate the differences in gelation, the selection of fish species with entirely different abilities for "setting" would be useful, thus flying fish and black marlin muscles were used. The purpose of this study was to clarify the mechanism of thermal activation of actomyosin Mg^{2+}-ATPase in order to elucidate the setting characteristics among fish species.

MATERIALS AND METHODS

Materials

The disodium salt of ATP, trypsin, trypsin inhibitor (soybean type 1-S), chymotrypsin, phenylmethyl-sulfonyl, and fluoride (PMSF) were purchased from Sigma Chemical Co. Ltd. All other chemicals used were reagent grade.

Flying fish (*Gypselurus opisthopus*) was used as the more easy-setting protein and black marlin (*Makaira mazara*) as the difficult-setting protein.

The authors are with the Dept. of Food Engineering, Faculty of Fisheries, Tokyo Univ. of Fisheries, Konan, Minato-ku, Tokyo 108, Japan.

Sample preparation

For the preparation of meat pastes, each fish meat was minced in a chopper (pore size 3 mm) and the minced meat washed with cold water to remove blood, fat, and water soluble components. The washed meat was centrifuged, adjusted to a moisture content of 84–85% (net basis), and to pH 6.8–6.9 with the addition of 0.1N NaOH. The meat and 2.5% NaCl added by weight were ground into a meat paste, packed in stainless tubes (diameter 3.0 cm × height 3.0 cm) and heated at 30°C and 40°C for 10, 20, 40, and 60 min. The gels formed in the tubes after heat treatment were subjected to jelly strength measurement with a rheometer (Type R-UDJ, San Kagaku Co. Ltd., plunger diameter, 0.5 cm). The value of jelly strength was expressed as the product of breaking load and breaking strain (g·cm).

For the preparation of muscle protein, the dorsal muscle of flying fish and black marlin was carefully excised. Myosin was prepared from both fish muscles by the method of Mackie and Connell (1964) with some modifications. The myosin solutions, containing 0.5M KCl, 5 mM $MgCl_2$, 5 mM ATP, 20 mM Tris-maleate buffer (pH 7.5), were clarified by ultracentrifugation at 100,000 × *g* for 3 hr by the method of Weber (1956), and then each myosin was collected by fractional precipitation (40–55% ammonium sulfate saturation).

Actin was prepared from the acetone-dried muscles by the method of Spudich and Watt (1971) and transformed into F-actin by polymerization in 0.1M KCl and 1 mM $MgCl_2$.

For the preparation of heavy meromyosin (HMM), each myosin solution (8.0 mg/mL) in 0.5M KCl, 20 mM Tris-maleate (pH 7.5) was digested at 10°C for 10 min with trypsin (1/300, W/W) relative to myosin (Kimura et al., 1979, 1980). The digestion was stopped by adding trypsin inhibitor in a 5-fold excess over trypsin (W/W). The digest was dialyzed overnight at 0–4°C against 10 vol. 10 mM Tris-maleate buffer (pH 7.0), centrifuged (100,000 × g) for 1 hr, and then subjected to fractionation by ammonium sulfate (giving 45-55 % saturation) to obtain HMM fraction. The fraction was dialyzed against 10 mM Tris-maleate buffer, pH 7.5, and centrifuged at 100,000 × *g* for 1 hr. The supernatant was used as the HMM.

Each subfragment-1 (S-1) was prepared by the method described by Ikariya et al. (1981). The digestion was performed at 10°C for 20 min with chymotrypsin in an amount of 1/130 (W/W) relative to myosin (10 mg/mL) in 0.5M KCl, 20 mM Tris-maleate, pH 7.5. After stopping the digestion by the addition of PMSF (final concentration, 0.2 mM), the digest was dialyzed against 10 vol. 10 mM Tris-maleate buffer (pH 7.5). The dialyzed digest was centrifuged at 50,000 × *g* for 60 min and the supernatant was subjected to gel filtration on a Sephadex G-200 column (2.2 × 88 cm) equilibrated with 0.05M KCl and 20 mM Tris-maleate (pH 7.5). Gel filtration was carried out at 4°C and eluted with the same buffer. The flow rate was about 12 mL/hr. Fractions of 4.8 mL were collected and absorbance at 280 nm measured. The fraction at 1.54 Ve/Vo (elution volume/void volume) was used as S-1. SDS-gel electrophoresis was carried out according to Weber and Osborn (1969). The gels (0.5 cm in diameter and 10 cm in length) of 10% polyacrylamide and 0.1% SDS were used. After the run, the protein bands were stained with Coomassie brilliant blue.

Assay of ATPase activity

The actomyosin, acto-HMM, and acto-S-1 were made up of 3 mg/mL myosin or HMM, 0.3 mg/mL S-1, and F-actin in an amount equal to myosin, HMM, or S-1 (protein weight), respectively. They were incubated at 25°, 30°, 35°, 37°, or 40°C for 10 min unless otherwise specified, in 0.1M KCl and 20 mM Tris-maleate, pH 7.0. Incubation was stopped by cooling in ice-water. ATPase activity was assayed at 25°C for 2 min, after 5 min preincubation. The reaction mixture (2 mL) contained 1 mM $MgCl_2$, 0.03M KCl, 20 mM Tris-maleate (pH 7.0) and 0.3 mg/mL actomyosin, 0.3 mg/mL acto-HMM, or 0.1 mg/mL acto-S-1. The reaction was started by the addition of 2 mM ATP and stopped by adding 2 mL 10% trichloroacetic acid. The mixture was centrifuged at 3,000 × *g* for 5 min, and the supernatant analyzed for liberated inorganic phosphate (Pi) by the method of Martin and

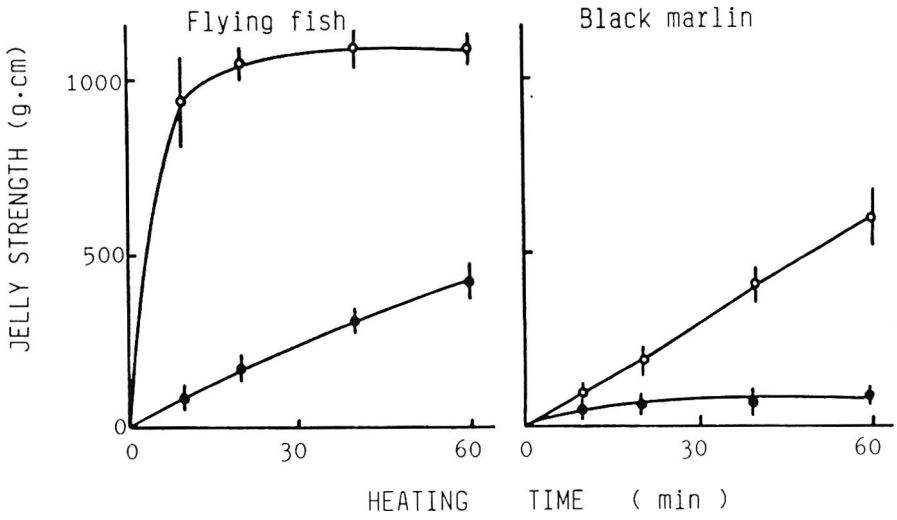

Fig. 1—Time courses of thermally induced gelation of flying fish and black marlin meat pastes. Temperatures for the meat paste gelation were 30°C. (●) and 40°C (○).

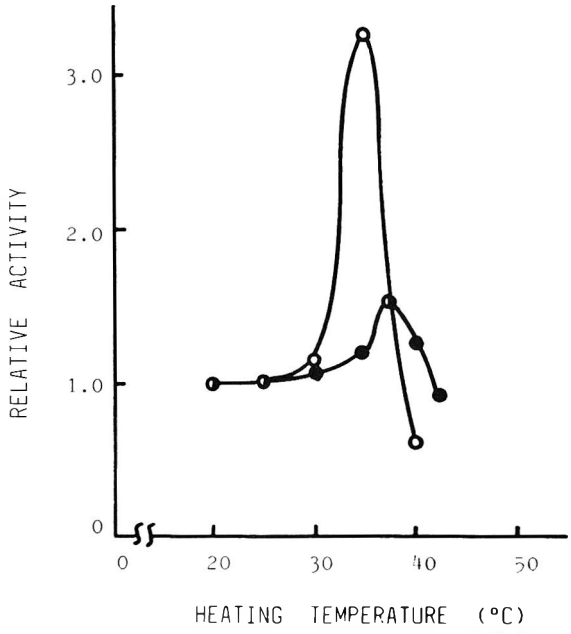

Fig. 2—Changes in the activity of actomyosin Mg^{2+}-ATPases due to thermal treatment. Flying fish (○) and black marlin (●) actomyosins (1.0 mg/mL) were treated in the presence of 0.1M KCl-10 mM Tris-maleate buffer (pH 7.0) at the given tempertures for 10 min. The relative activity was expressed as the activity after thermal treatment/original activity.

Doty (1949). Protein concentration was determined by the biuret method (Gornall et al., 1949), and in S-1 concentration = 7.7 (Onodera et al., 1971).

RESULTS & DISCUSSION

TIME COURSES of thermally induced gelation of flying fish and black marlin meat pastes at 30° and 40°C are shown in Fig. 1. It was indicated from these curves that the initial velocity of gelation of flying fish was higher than that of black marlin at both temperatures. The difference observed in initial velocity is probably due to the reactivity of protein sensitive to heat, such as myosin and actomyosin, since these proteins are the major proteins in the thermally formed gel. It is well known that the potential of high initial velocity is essential for the easy-setting function of fish meat paste.

Figure 2 shows the changes in the activity of actomyosin Mg^{2-}-ATPase from flying fish and black marlin muscles due to thermal treatment. Relative activity versus treatment temperature profiles gave a maximum value at 35°C for flying fish and 37°C for black marlin. The maximum activation for flying fish was more than three times the original activity. For black marlin the activation was about one and a half times. Thus, differences in thermal activation were observed between the two fishes. If the thermal activation is closely related to the interaction between myosin and actin, the factors affecting the interaction may play an important role for differences in activation.

The time courses of actomyosin Mg^{2+}-ATPase incubated in the presence of various KCl concentrations are shown in Fig. 3. The thermal activation of flying fish actomyosin was affected remarkably by KCl concentration. The presence of 0.1M KCl increased the relative activity more than three times; in 0.3M KCl the activation increased about one and a half times and in 0.6M KCl only inactivation was observed. For black marlin the increase in the thermal activity was very low compared with that for flying fish, but activation in 0.1M KCl was the greatest. Since it is known that in presence of a high KCl concentration myosin is loosely bound to actin, it seems that, by being dependent on the interaction between myosin and actin, the thermal activation of the ATPase is closely related to the concentration of KCl.

The thermal activation of flying fish actomyosin Mg^{2+}-ATPase was not affected by the presence or absence of Ca^{2-}. This implies that the reaction occurring at 35°C was independent on the possible presence of minor regulatory components.

The changes in the activity of flying fish actomyosin Mg^{2+}-ATPases synthesized with thermally treated myosin or actin are shown in Fig. 4. When actin was treated below 42°C actomyosin Mg^{2-}-ATPase exhibited almost unchanged activities. In the case of thermally treated myosin, the activities began to decrease at temperatures as low as 25°C, suggesting that the thermal stability of myosin was low, compared to that of actomyosin. The heating of the myosin or actin did not cause any activation of actomyosin Mg^{2-}-ATPase. It would appear likely that the increase in activity was caused by the interaction between myosin and actin during thermal treatment and that such interaction did not occur in presence of the thermally treated myosin and actin, presumably because these proteins lose the ability to form actomyosin.

It was suggested that the myosin "tail" portions would be involved in the network formation for the gelation of rabbit muscle proteins (Samejima et al., 1981) and that the protein-protein reaction during setting of fish sol would result in the elastic texture of final products (Lanier et al., 1982). It is assumed, therefore, that a clue to elucidating the thermal activation of actomyosin Mg^{2-}-ATPase may be closely related

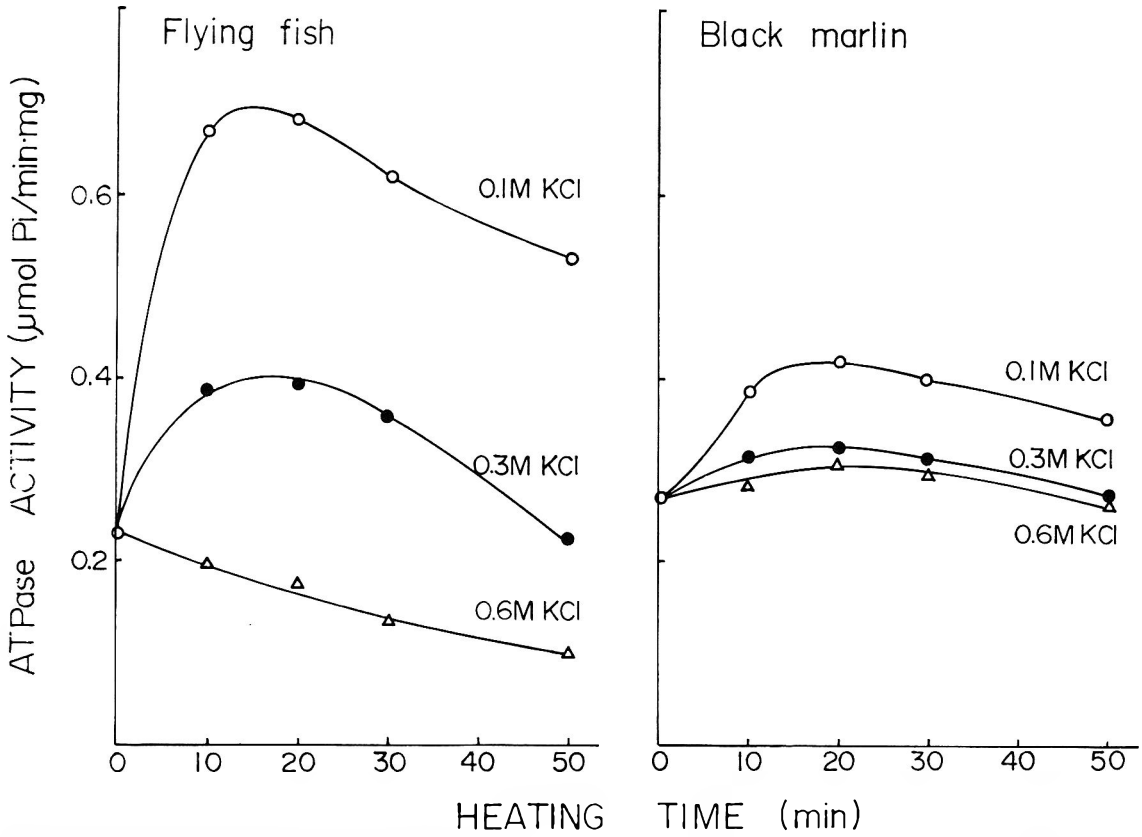

Fig. 3—Time courses of actomyosin Mg^{2+}-ATPases incubated in the presence of various KCl concentrations. Flying fish actomyosin was incubated at 35°C and that of black marlin at 37°C.

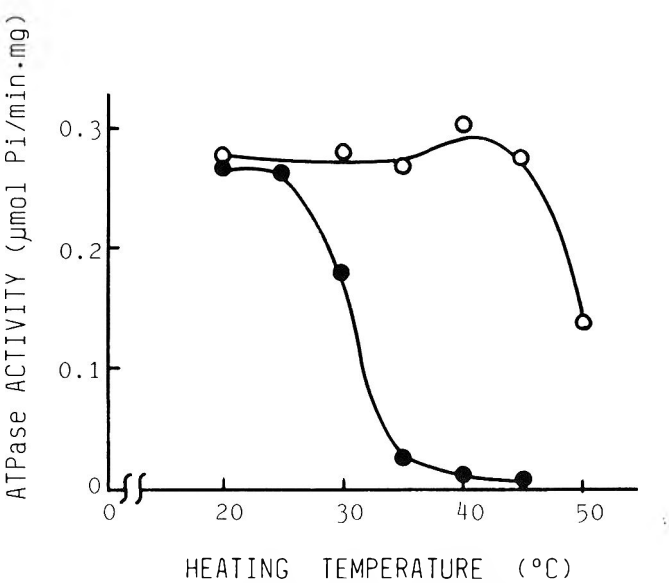

Fig. 4—Changes in the activity of actomyosin Mg^{2+}-ATPases synthesized from thermally treated myosin or F-actin. ○: Flying fish actomyosin was synthesized from untreated myosin and F-actin treated at the given temperatures for 10 min. ●: Flying fish actomyosin was synthesized from untreated F-actin and myosin treated at the given temperatures for 10 min. The ATPase activities were plotted against the treatment temperature of myosin or actin.

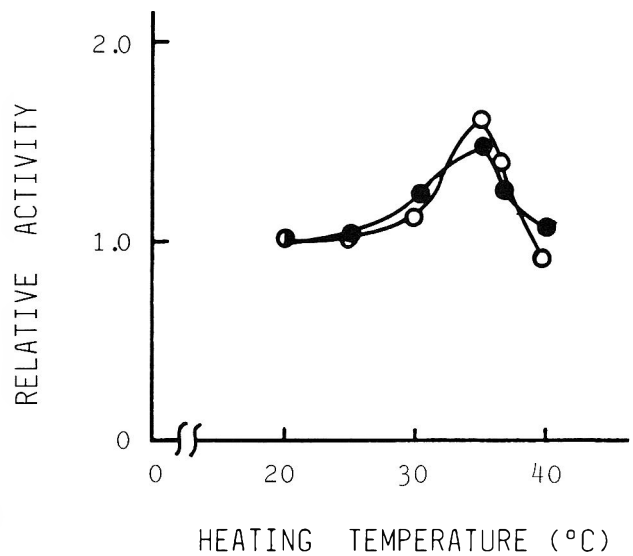

Fig. 5—Changes in the activity of acto-HMM Mg^{2+}-ATPases by thermal treatment. Flying fish (○) and black marlin (●) acto-HMM ATPases (1.0 mg/mL) in 0.1M KCl and 20 mM Tris-maleate (pH 7.0) were heated at the given temperatures for 10 min. The relative activity was expressed as the activity after thermal treatment/original activity.

to the interaction between myosin "tail" portions which occurred near 35°C. Under the above assumption, acto-HMM and acto-S-1 Mg^{2+}-ATPases were used in this experiment.

The increase in the activity of flying fish acto-HMM Mg^{2+}-ATPase, due to thermal treatment at 35°C, was approximately half that observed with actomyosin Mg^{2+}-ATPase (Fig. 5). In the case of black marlin, the relative activity of acto-HMM Mg^{2+}-ATPase was similar to that of actomyosin. Comparison of these data with Fig. 2 indicated that although distinct differences in thermal activation of actomyosin Mg^{2+}-ATPase activity were observed between flying fish and black marlin, there were no differences in that of acto-HMM Mg^{2+}-ATPase activity between the fishes. It is suggested from these results

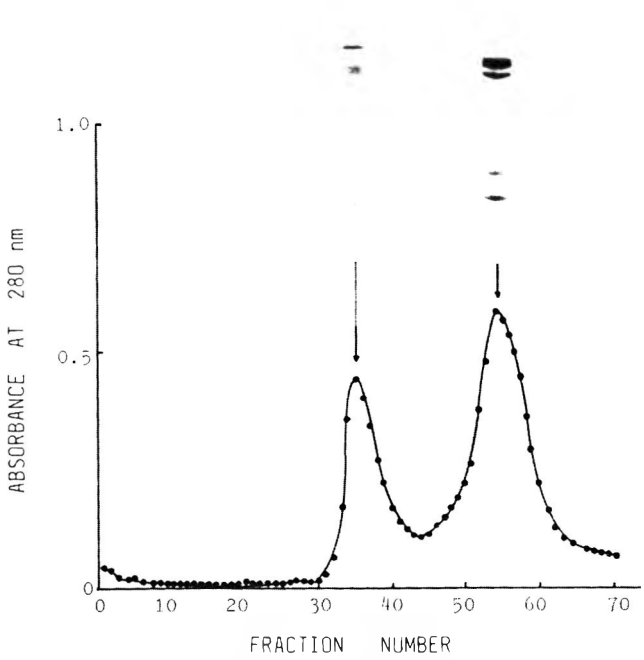

Fig. 6—Elution profile in Sephadex G-200 gel of S-1 fraction obtained by chymotryptic digest of flying fish myosin.

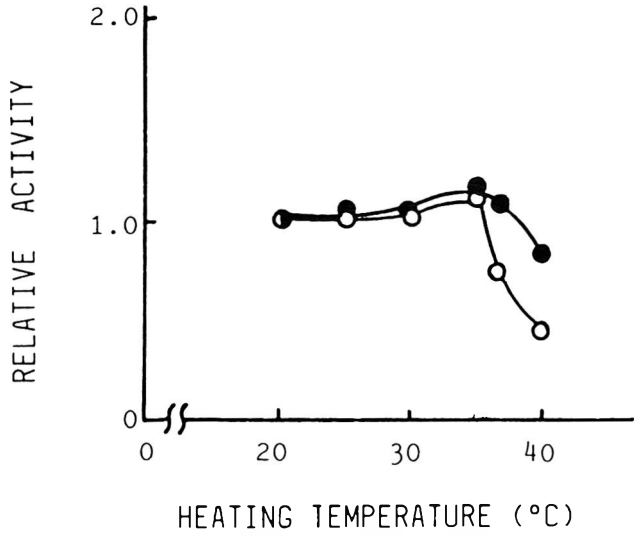

Fig. 7—Changes in the activity of acto-S-1 Mg^{2+}-ATPase due to heat treatment. Flying fish (○) and black marlin (●) acto-S-1 ATPases (0.1 mg/mL) were heated at the given temperatures for 10 min. The relative activity was expressed as the activity after thermal treatment/original activity.

that the maximum increase in the activity of flying fish actomyosin Mg^{2+}-ATPase by thermal treatment is very dependent on the myosin "tail" portion.

In order to explore further the possible role of the myosin "tail" portions in the actomyosin Mg^{2+}-ATPase, myosin subfragment-1 (S-1) was prepared from flying fish and black marlin myosins, respectively. Sephadex G-200 gel filtration curve of flying fish S-1 is shown in Fig. 6. SDS-gel electrophoresis of the two large peaks showed that the first peak in void volume comprised HMM and the second one, in 15.4 of elution volume ratio to void volume, was S-1. These results almost coincided with those of carp S-1 (Ikariya et al., 1981).

The changes in the activity of acto-S-1 Mg^{2+}-ATPases due to thermal treatment are given in Fig. 7. There was no increase in the activity of acto-S-1 Mg^{2+}-ATPase from flying fish and black marlin muscles over the range of treatment temperatures. Thus the activation of Mg^{2+}-ATPase by the thermal treatment showed a decrease in the following order: actomyosin > acto-HMM > acto-S-1. It seems, therefore, that the "rod" or "tail" portion of myosin plays an important role in the thermal activation of actomyosin Mg^{2+}-ATPase.

The setting ability of fish meat paste and the thermal activation of actomyosin Mg^{2+}-ATPase from flying fish were very high compared with those from black marlin. If the setting ability is dependent upon myosin molecule, it may be caused by heat changes of myosin "tail" portions. The study of the role of myosin "tail" portions in thermal gelation will be published in the near future.

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Tenderness and Retail Display Characteristics of Beef Tenderloin Steaks Comparing Kidney Fat Removal Times Under Different Processing and Storage Systems

D. D. JOHNSON, S. T. WEIFFENBACH, and R. L. WEST

ABSTRACT

The effects of electrical stimulation, kidney fat removal time, tenderloin removal time, and storage and packaging treatment on tenderness and retail display characteristics of beef tenderloin steaks were studied. Electrical stimulation increased overall desirability scores on days 2 through 4 of retail display. Steaks from sides that had kidney and pelvic fat removed prior to chilling had higher Warner-Bratzler shear force values, darker muscle color and higher retail evaluations for overall desirability on days 2 through 4 of display than did steaks from conventionally dressed sides. Tenderloin steaks that were vacuum packaged and stored 14 days had higher bacterial counts, more uniform lean color and less visual purge in the retail package than did steaks retail displayed immediately after tenderloin removal.

INTRODUCTION

THE AMERICAN MEAT INSTITUTE submitted a Citizens' Petition (Smalley, 1984) to the USDA in May, 1983 to amend the existing federal meat grading regulations to require that beef carcasses have most of the kidney, pelvic and heart (KPH) fat removed before being presented for official grading. This prompted a proposed rule change in the standards for grades of carcass beef by the Agricultural Marketing Service of USDA (Smalley, 1984) in November, 1984, allowing KPH removal prior to grading. Although the proposed rule change has been withdrawn, the effects of allowing KPH removal prior to grading are still in question.

Several reasons for the removal of KPH prior to grading have been brought to light during this proposed rule change. Approximately 60% of the fed beef produced in 1982 left the packer in the form of boxed beef, and this figure is predicted to rise to 90% by 1992. An impact study by Smalley (1984) suggested that approximately 72% of the KPH fat currently is removed at the packer in the fabrication process. These fats are usually rendered into tallow and therefore less energy would be required if these fats were rendered while still warm. Smalley (1984) suggested that as much as $16.3 million could be saved annually in labor, energy and transportation if KPH fats were removed prior to chilling and grading.

There are some concerns that have discouraged beef processors from removing KPH fat prior to chilling. One concern is that some packers and processors feel that there would be some pricing confusion when merchandising carcasses and cuts with kidney fat removed in comparison to carcasses and cuts in which KPH fat is left intact (Anonymous, 1985). Currently, carcasses that have substantial KPH fat removed prior to presentation for grading must be identified for grade by applying the roller brand in reverse, and generally these carcasses are regarded as inferior and are often discounted in price (Smalley, 1984). Another concern is that KPH fat removal could potentially expose the beef tenderloin to damage and deterioration.

Much of the interest, to date, has been directed toward the effects that removal of kidney and pelvic fat prior to chilling would have on the current USDA yield grading standards. However, little research has been conducted to determine the effects of kidney and pelvic fat removal prior to chilling on the quality characteristics of tenderloins which are normally protected by this fat. De Felicio et al. (1982) reported that removal of kidney fat before chilling caused the psoas major muscle to be darker in color at days 0 and 1 of retail display, but lean color was not different from the conventionally treated beef at days 2 and 4. These researchers also noted slightly higher shear force values for steaks from sides which had kidney fat removed before chilling than for those treated conventionally.

A change in carcass dressing procedures that possibly could cause changes to a carcass component as valuable as the beef tenderloin would warrant further examination. This study was designed to explore further the effects of KPH removal prior to chilling and subsequent situations that might occur in the normal processing of beef.

MATERIALS & METHODS

TWENTY-FOUR CROSSBRED steer carcasses were utilized in this study. One side of each carcass was electrically stimulated (ES) within 1 hr after bleeding, with 500 volts for 20 × 2 sec pulses, whereas the other side was not electrically stimulated (NS). At random, one-half of the ES and NS sides had the kidney and pelvic fat substantially removed on the slaughter floor prior to chilling (ACC) and the other one-half of the beef sides were handled in a conventional manner (CONV) in which the kidney fat remained intact during chilling. All carcasses were chilled at 1° ± 1°C in a blast cooler.

Beef sides within kidney fat removal treatment were then randomly assigned to one of two tenderloin removal treatments. The first treatment where the full tenderloin was removed at 48 hr (2d) represented those packers who produce boxed beef and normally fabricate at the same location that the animals were slaughtered. The other tenderloin removal treatment represented those packers who ship carcasses or quarters to other locations for fabrication or those retailers buying carcass beef, which involved tenderloin removal after 144 hr (6d). At the time of tenderloin removal, tenderloins were cut in half and the anterior or posterior halves randomly assigned to one of two packaging and storage treatments. One treatment was designated as a control (CONT) from which three steaks were removed from the respective one-half of the tenderloin. One steak was used for cooking characteristics and Warner-Bratzler shear force determinations, one steak was used for sarcomere length and fragmentation index measurements, and the third was placed in a foam tray wrapped in polyvinyl chloride film and subsequently evaluated for retail display characteristics. All steaks except those for retail display were frozen at − 18°C until laboratory determinations could be performed. The second storage treatment consisted of vacuum packaging the remaining one-half of the tenderloin in a Cryovac® B620 barrier bag (VAC) and storing at 1° ± 1°C for 14 days, then steaks were prepared in a manner similar to the CONT portion of the tenderloin.

Steaks for Warner-Bratzler shear (WBS) analysis were thawed 18 hr at 2–4°C, then broiled on Farberware Open-Hearth broilers to an internal temperature of 70°C (AMSA, 1978). Internal temperatures were monitored using copper-constantan thermocouples attached to a Leeds and Northrup Speedomax®-165 potentiometer. After steaks were cooled to 21°C, eight cores (1.27 cm diameter) were removed parallel to fiber orientation and sheared on a Warner-Bratzler shearing device.

Sarcomere length was determined by homogenizing 5g of sample

The authors are with the Animal Science Dept., 224 Animal Science Bldg., Univ. of Florida, Gainesville, FL 32611.

in 25 mL of cold 0.25M sucrose solution. A drop of homogenate was placed on a microscope slide and covered with a cover slip. The slide was placed on a stage and a helium-neon laser (Model 155, Spectra Physics, Inc., Mt. View, CA) light (0.95 mW) was shown through individual myofibrils. Fifteen diffraction patterns were measured and the equation of Cross et al. (1980) was used to convert the measurements to sarcomere length in micrometers.

Fragmentation index was performed according to the procedure outlined by Davis et al. (1980). This involved homogenizing 10g of meat in 50 ml of cold homogenizing solution (0.25 M sucrose, 0.02M KCl) at full speed for 78 sec using a Virtis "23" homogenizer (The Virtis Co., Gardiner, NY). The homogenate then was filtered through a 250 μm mesh screen. After a 10 min drying period, the screens were weighed and fragmentation index was calculated according to Davis et al. (1980).

Tenderloin steaks were placed in plastic foam trays and overwrapped in polyvinyl chloride film (Oxygen Transmission Rate = 6,500 cc/m^2/24 hr at 0% relative humidity). Steaks were retail displayed in Nolin (model OVFM, Nolin Manufacturing Co., Montgomery, AL) retail cases at an air temperature of 2 ± 3°C. All steaks were displayed in random order under GE "cool white" fluorescent light following commercial time patterns of lighting (12 hr on, 12 hr off). A six member experienced panel evaluated each sample utilizing either a four or eight point descriptive scale for overall desirability (1 = extremely undesirable; 8 = extremely desirable), muscle color (1 = very dark red; 8 = very light cherry red), surface discoloration (1 = 100% discolored; 8 = no discoloration), muscle color uniformity (1 = uniform color; 4 = dissimilar color) and visual purge (1 = none; 4 = extreme amount).

Aerobic plate counts (APC) were determined by aseptically removing 20–25g meat samples (2 mm thick) from several locations on the surface of the steaks after they were removed from retail display. Meat samples were serially diluted (1:10) with sterile Butterfield's phosphate diluent in Stomacher bags and macerated for 1 min in a Stomacher-400. Bacterial counts were then determined by plating 0.5 mL of diluent on pre-poured Baltimore Biological Laboratories Standard Methods Agar plates at appropriate dilutions (APHA, 1984) then incubated for 5 days at 20°C. Plate counts were converted to $\log_{10}$ values for data analysis.

Data were analyzed as a 2^4 factorial design with electrical stimulation, kidney fat removal, tenderloin removal, and packaging and storage serving as treatment main effects, using the general linear model of the Statistical Analysis System (SAS, 1982). F-tests were used to determine the significance of main effects and interactions means were separated using Least Significant Differences (Steel and Torrie, 1980).

RESULTS & DISCUSSION

MAIN EFFECT mean values for aerobic plate counts (APC) of tenderloin steaks sampled after retail display are presented in Table 1. An electrical stimulation X time of tenderloin removal interaction ($P<0.05$) was found and these mean values are given in Table 2. A greater difference in $\log_{10}$ APC between steaks from tenderloins removed at 2d versus 6d was encountered for the electrically stimulated sides in comparison to the steaks from sides not receiving electrical stimulation where no significant differences were noted between 2d and 6d tenderloin removal treatments. Removing the KPH fat prior to chilling (ACC) did to affect $\log_{10}$ bacterial counts statistically, although CONV tenderloin steaks were almost one-half $\log_{10}$ higher in bacterial counts. The significant increase in $\log_{10}$ APC for the VAC tenderloin steaks as compared to the CONT steaks would be expected due to the bacterial growth that occurred during the two week period the VAC steaks were stored before retail display.

Warner-Bratzler shear force values were higher than those reported by de Felicio et al. (1982) which could have been partially due to the slightly more drastic cooking parameters in the present study. A significant ($P<0.05$) three-way interaction between electrical stimulation treatment × time of tenderloin removal × packaging and storage treatment was noted for WBS values (Table 3). The interpretation of this three-way interaction was not readily apparent. In general, steaks from ES sides that had the tenderloins removed at 2d were more tender ($P<0.05$) than steaks from NS sides that had tenderloins also removed at 2d. Steaks from ES sides with tenderloins removed at 6d had significantly higher ($P<0.05$) WBS values than did steaks from ES sides with tenderloins removed at 2d, which was unexpected due to the shorter postmortem aging that would have occurred in the 2d steaks. All other treatment combinations were not statistically different in WBS values. Steaks from ACC beef sides had significantly higher WBS values than steaks from CONT sides; however, values were in the tender range.

No treatment differences were noted for sarcomere length, fragmentation index or thaw loss (Table 1). Sarcomere length data suggested that the exposed muscles in the tenderloins of the ACC treatment underwent little cold shortening during chilling, perhaps due to the remaining attachments to the carcass. Steaks from beef sides with KPH fat removed prior to chilling and grading (ACC) had less cooking loss than did steaks from beef sides with the KPH fat left intact until fabrication (CONV). This possibly could be due to the dehydration occurring during chilling and storage of the ACC beef sides.

Electrical stimulation had only a small effect on visual color scores of tenderloin steaks during retail display (Table 4). Tenderloin steaks from ES sides were rated as lighter ($P<0.05$) in color on day 1 than were tenderloin steaks from NS sides. On all other days of retail display, steaks from ES sides did not differ statistically in lean color from steaks from NS sides. Time of kidney fat removal did affect muscle color in that lean color of the steaks from the ACC sides was significantly darker ($P<0.05$) than that of steaks from CONV sides. These findings were consistent with those of de Felicio et al. (1982) who reported that "unprotected tenderloins" had faster rates of temperature decline than did tenderloins protected by perine-

Table 1—Mean values for aerobic plate counts, Warner-Bratzler shear force, cooking characteristics and histological measurements by treatment main effects

Trait	Electrical stimulation[a]		Kidney fat removal[b]		Tenderloin removal[c]		Packaging and storage[d]	
	NS	ES	ACC	CONV	2d	6d	CONT	VAC
Aerobic plate count ($\log_{10}$/g)	5.85	5.98	5.68	6.14	5.55[e]	6.28[f]	4.91[e]	6.92[f]
Warner-Bratzler shear force (kg/1.27 cm)	3.59	3.36	3.61[e]	3.35[f]	3.45	3.50	3.60[e]	3.36[f]
Sarcomere length (μm)	3.55	3.51	3.51	3.55	3.54	3.52	3.55	3.51
Fragmentation index	570.5	565.5	559.0	557.0	567.7	568.2	575.3	562.6
Thaw loss (%)	0.42	0.25	0.24	0.43	0.22	0.46	0.44	.23
Cook loss (%)	29.02	28.72	27.74[e]	30.00[f]	28.89	28.85	29.41	28.33

[a] NS = not stimulated; ES = electrically stimulated.

[b] ACC = kidney and pelvic fat substantially removed during slaughter sequence; CONV = kidney fat removal at the time of tenderloin removal.

[c] 2d = tenderloin removed after 48 hr chill at 1 ± 1°C; 6d = tenderloin removed after 144 hr chill at 1 ± 1°C.

[d] CONT = immediately after the tenderloin was removed from the carcass 2.54 cm steaks were removed for analysis; VAC = after the tenderloin was removed from the carcass, at random, one-half of the tenderloin was placed in a barrier bag and held at 1 ± 1°C for 14 days and subsequently treated as the CONT.

[e,f] Means within the same row and main effect with different superscripts differ ($P<0.05$).

Table 2—Means for aerobic plate counts for electrical stimulation treatment × time of tenderloin removal

Trait	Not stimulated		Electrically stimulated	
	2d[a]	6d	2d	6d
Aerobic plate counts (log_{10}/g)	5.77[bc]	5.94[c]	5.32[b]	6.63[cd]

[a] 2d = tenderloin was removed after 48 hr chill at 1° ± 1°C; 6d = tenderloin was removed after 144 hr chill at 1 ± 1°C.
[b,c,d] Means bearing a common superscript letter are not different.

phric fat during postmortem chilling, and subsequently darker lean color on days 0 and 1 of display. Several researchers have suggested that rate of postmortem glycolysis can be slowed by increasing the rate of muscle temperature decline (Marsh, 1954; Taylor et al., 1980). A slower rate of postmortem glycolysis may be responsible for the darker lean color reported in the present study. Packaging and storage treatment had no effect on lean color except on day 1 where a two-way interaction ($P<0.05$) between kidney fat removal treatment and the packaging and storage method was encountered (Table 5). Tenderloin steaks from CONV sides, regardless of the packaging and storage treatment, were lighter in color than ACC sides. No differences in muscle color between steaks from CONT versus VAC were noted for the sides chilled with the KPH fat intact, but tenderloin steaks from sides that were chilled with the kidney fat removed and immediately displayed were darker ($P<0.05$) in color than steaks from ACC sides that were placed in vacuum storage for 2 wk. This suggested that lean color might become lighter during vacuum storage, but the lean would still remain darker on day 1 of retail display than would be expected of tenderloin steaks from CONV processed beef carcasses.

In addition, steaks from tenderloins removed at 6d were scored lighter in lean color on day 1 through day 4 of retail display than were steaks from tenderloins removed at 2d (Table 4). A two-way interaction ($P<0.05$) between time of tenderloin removal and packaging and storage was noted for muscle color on day 4 (Table 6). At day 4 of retail display the steaks from tenderloins removed at 2d were darker in lean color, regardless of packaging and storage treatment, than steaks from tenderloins removed at 6d and immediately displayed (CONT). In contrast, steaks from beef sides in which the tenderloins were removed at 6d and vacuum packaged for 2 wk (VAC) were intermediate in lean color and not significantly different from the other three treatment combinations.

Electrical stimulation had only a small effect on surface discoloration on days 0 and 1 (Table 4), although steaks from ES sides had less surface discoloration on days 2 and 3 of the retail display period. These results were in agreement with findings of Hall et al. (1980) who reported that beef round steaks from ES sides had less surface discoloration over a 4-day retail display period than steaks from their nonstimulated counterparts.

Removing kidney fat prior to chilling did affect ($P<0.05$) the rate of surface discoloration in the present study in that on day 1 through day 4 steaks from ACC sides had significantly less surface discoloration than steaks from CONV sides. However, the dark color of the steaks from the ACC treatment may have masked the discoloration.

Steaks from tenderloins removed at 6d had more ($P<0.05$) surface discoloration than did steaks from tenderloins removed at 2d, but time of tenderloin removal had no effect ($P>0.05$) on the amount of discoloration found at day 1. Similar results were noted for surface discoloration effects due to packaging and storage treatment in that CONT steaks had more surface discoloration on day 0 than did VAC steaks, although no effects due to packaging and storage treatment were found on day 1. A significant interaction for time of tenderloin removal × packaging and storage treatment interaction ($P<0.05$) was encountered for day 2 through day 4 for surface discoloration (Table 6). On days 2 and 3 of retail display, steaks from 6d sides and retail displayed immediately (CONT) had the least amount of surface discoloration, in contrast steaks from 2d sides which were placed immediately into the retail display (CONT) had the most discoloration (Table 6). Steaks from tenderloins removed at 2d or 6d and vacuum packaged for two weeks (VAC) before retail display were intermediate in discoloration to the other two treatment combinations. On day 4, VAC steaks from sides that had the tenderloin removed at 6d had more ($P<0.05$) surface discoloration than did VAC steaks from sides that had tenderloins removed at 2d. The CONT steaks from the 2d and 6d tenderloin removal treatments were intermediate in surface discoloration to the two VAC package and storage treatments. Steaks from tenderloins removed at 6d and vacuum packaged for 14 days had the greatest time span for microbial deterioration which probably was responsible for the amount of surface discoloration encountered.

Steaks from sides that were not electrically stimulated had more uniform muscle color than did steaks from ES sides at the initial (day 0) retail evaluation, but color uniformity was not affected by ES treatment on day 1 through day 4 of the retail display period (Table 4). Time of kidney fat removal had no apparent influence on muscle color uniformity during the retail display evaluation period. Steaks from tenderloins removed at 2d were not as uniform in muscle color on days 1 through 3 of retail display as were steaks from tenderloins removed at 6d. Also, steaks from the CONT packaging and storage tretment had less ($P<0.05$) uniform muscle color than did steaks from the VAC packaging and storage treatment for the entire retail evaluation period. A two-way interaction ($P<0.05$) between time of tenderloin removal and packaging and storage treatment was noted on days 2 and 3 of retail display for muscle color uniformity (Table 6). This interaction occurred due to the greater difference in muscle color uniformity scores between the steaks from the CONT and VAC packaging and storage treatment for tenderloin removed at 2d than was found between CONT and VAC steaks from tenderloins removed at 6d.

Visual purge scores during retail display were not affected by electrical stimulation or time of tenderloin removal as shown in Table 4. In addition, kidney fat removal treatment had no effect on visual purge scores on days 0 through 3 of the retail display period, but on day 4 of retail display steaks from ACC treated sides had more ($P<0.05$) visual purge than did steaks from CONT sides. The most consistent differences in visual

Table 3—Mean values for Warner-Bratzler shear force for electrical stimulation × tenderloin removal × packaging and storage treatments

Trait	Not stimulated				Electrically stimulated			
	2d[a]		6d		2d		6d	
	CONT[b]	VAC	CONT	VAC	CONT	VAC	CONT	VAC
Warner-Bratzler shear force (kg/1.27 cm)	3.87[d]	3.51[cd]	3.51[cd]	3.46[cd]	3.20[c]	3.24[cd]	3.80[d]	3.23[c]

[a] 2d = tenderloin removed after 48 hr chill at 1 ± 1°C; 6d = tenderloin removed after 144 hr chill at 1 ± 1°C.
[b] CONT = immediately after the tenderloin was removed from the carcass 2.54 cm steaks were removed for analysis; VAC = after the tenderloin was removed from the carcass, at random, one-half of the tenderloin was placed in a Cryovac barier bag and held at 1° ± 1°C for 14 days and subsequently treated as CONT.
[c,d] Means with different superscripts differ ($P<0.05$).

Table 4—Mean values for retail evaluations of muscle color, discoloration, muscle color uniformity, visual purge, and overall desirability of beef tenderloin steaks by treatment main effects

	Electrical stimulation[a]		Kidney fat removal[b]		Tenderloin removal[c]		Packaging and storage[d]	
Trait	NS	ES	ACC	CONV	2d	6d	CONT	VAC
Muscle color[e]								
Day 0	4.77	5.00	4.28	5.50	4.80	4.97	4.85	4.92
Day 1	4.73[j]	5.04[k]	4.27[j]	5.50[k]	4.69[j]	5.09[k]	4.81	4.97
Day 2	4.46	4.76	4.08[j]	5.14[k]	4.37[j]	4.85[k]	4.50	4.71
Day 3	4.45	4.81	4.06[j]	5.20[k]	4.30[j]	4.96[k]	4.64	4.62
Day 4	4.28	4.49	3.86[j]	4.91[k]	4.13[j]	4.65[k]	4.44	4.33
Discoloration[f]								
Day 0	7.66	7.61	7.60	7.67	7.71[j]	7.56[k]	7.47[j]	7.80[k]
Day 1	6.47	6.61	6.73[j]	6.35[k]	6.44	6.64	6.60	6.48
Day 2	4.94[j]	5.41[k]	5.75[j]	4.59[k]	4.93[j]	5.41[k]	5.18	5.16
Day 3	4.18[j]	4.75[k]	5.15[j]	3.78[k]	4.29	4.65	4.38	4.55
Day 4	3.85	4.09	4.70[j]	3.23[k]	4.09	3.84	3.94	4.00
Muscle color uniformity[g]								
Day 0	1.35[j]	1.48[k]	1.45	1.38	1.39	1.44	1.50[j]	1.33[k]
Day 1	1.47	1.56	1.52	1.51	1.58[j]	1.45[k]	1.73[j]	1.30[k]
Day 2	1.70	1.68	1.70	1.68	1.81[j]	1.57[k]	1.88[j]	1.50[k]
Day 3	1.69	1.69	1.72	1.66	1.78[j]	1.61[k]	1.90[j]	1.48[k]
Day 4	1.69	1.71	1.73	1.67	1.75	1.66	1.88[j]	1.53[k]
Visual purge[h]								
Day 0	1.00	1.00	1.00	1.01	1.01	1.00	1.00	1.00
Day 1	1.14	1.16	1.17	1.14	1.18	1.13	1.29[j]	1.02[k]
Day 2	1.21	1.22	1.22	1.20	1.22	1.20	1.41[j]	1.02[k]
Day 3	1.18	1.17	1.22	1.12	1.20	1.15	1.32[j]	1.03[k]
Day 4	1.12	1.14	1.22[j]	1.04[k]	1.17	1.08	1.23[j]	1.03[k]
Overall desirability[i]								
Day 0	6.78	6.78	6.56[j]	7.01[k]	6.77	6.79	6.35[j]	7.21[k]
Day 1	5.42	5.80	5.62	5.61	5.33[j]	5.90[k]	5.39[j]	5.84[k]
Day 2	4.22[j]	4.73[k]	4.81[j]	4.14[k]	4.35	4.61	4.48	4.47
Day 3	3.60[j]	4.08[k]	4.20[j]	3.48[k]	3.89	3.79	3.98	3.69
Day 4	3.10[j]	3.60[k]	3.82[j]	2.88[k]	3.53	3.17	3.50	3.20

[a] NS = not stimulated; ES = electrically stimulated.
[b] ACC = all kidney fat removed during slaughter sequence; CONV = kidney fat removed at the time of tenderloin removal.
[c] 2d = tenderloin removed after 48 hr chill at 1° ± 1°C; 6d = tenderloin removed after 144 hr chill at 1° ± 1°C.
[d] CONT = immediately after the tenderloin was removed from the carcass a (2.54 cm) steak was removed and placed in foam tray and PVC overwrapped and retail displayed; VAC = after the tenderloin was removed from the carcass it was placed in a barrier bag and held at 1° ± 1°C for 14 days and subsequently treated as the "control".
[e] 1 = very dark red; 8 = very light cherry red.
[f] 1 = 100% discoloration; 8 = no discoloration.
[g] 1 = uniform color; 4 = dissimilar color.
[h] 1 = none; 4 = extreme amount.
[i] 1 = extremely undesirable; 8 = extremely desirable.
[j,k] 1 = Means within the same row and main effect with different superscripts differ (P<0.05).

Table 5—Mean values for muscle color by kidney fat removal × packaging and storage treatments

	Accelerated		Conventional	
Trait	CONT[a]	VAC[a]	CONT	VAC
Muscle color[b] Day 1	4.06[c]	4.48[d]	5.55[e]	5.46[e]

[a] CONT = immediately after the tenderloin was removed from the carcass a 2.54 cm steak was removed and placed in a foam tray and PVC overwrapped and retail displayed; VAC = after the tenderloin was removed from the carcass, at random, one-half of the tenderloin was placed in a barrier bag and held at 1° ± 1°C for 14 days and subsequently treated as the CONT.
[b] 1 = very dark red; 8 = very light red.
[c,d,e] Means with different superscripts differ (P<0.05).

Table 6—Mean values for muscle color, discoloration, muscle color uniformity and visual purge by time of tenderloin removal × packaging and storage treatments

	2d[a]		6d[a]	
Trait	CONT[b]	VAC[b]	CONT	VAC
Muscle color[c]				
Day 4	4.03[g]	4.22[g]	4.85[h]	4.44[gh]
Discoloration[d]				
Day 2	4.62[g]	5.24[hi]	5.74[i]	5.08[gh]
Day 3	3.76[g]	4.81[hi]	5.00[i]	4.29[gh]
Day 4	3.82[gh]	4.36[h]	4.06[gh]	3.63[gh]
Muscle color uniformity[e]				
Day 2	2.09[i]	1.53[gh]	1.67[h]	1.47[g]
Day 3	2.08[i]	1.47[g]	1.71[h]	1.50[g]
Visual purge[f]				
Day 1	1.39[i]	0.97[g]	1.19[hi]	1.06[gh]
Day 2	1.50[i]	0.94[g]	1.31[hi]	1.09[gh]
Day 3	1.41[i]	0.97[g]	1.22[hi]	1.08[gh]

[a] 2d = tenderloin removed after 48 hr chill at 1° ± 1°C; 6d = tenderloin removed after 144 hr chill at 1° ± 1°C.
[b] CONT = immediately after the tenderloin was removed from the carcass a (2.54 cm) steak was removed and placed in foam tray and PVC overwrapped and retail displayed; VAC = after the tenderloin was removed from the carcass it was placed in a barrier bag and held at 1° ± 1°C for 14 days and subsequently treated as the "control".
[c] 1 = very dark red; 8 = very light cherry red.
[d] 1 = 100% discoloration; 8 = no discoloration.
[e] 1 = uniform color; 4 = dissimilar color.
[f] 1 = none; 4 = extreme amount.
[g,h,i] 1 = Means within the same row bearing a common superscript letter are not different (P<0.05).

purge scores were encountered for the packaging and storage treatment, in that on days 1 through 4 of the display period CONT steaks had more visual purge than did VAC steaks. Lower purge scores for the VAC steaks were probably due to purge lost in the vacuum bag as a result of the 14 days additional postmortem aging of the VAC steaks.

Electrical stimulation had no significant effect on overall desirability (P>0.05) on days 0 and 1 of retail display; however, steaks from ES sides were rated higher in overall desirability on day 2 through day 4. Hall et al. (1980) reported higher overall appearance scores for top round steaks from electrically stimulated sides on day 1 and day 3 of retail display than were noted for steaks from non-stimulated sides. The findings from the present study, plus findings from Hall et al. (1980) suggest that electrical stimulation may increase shelf life as much as 12 hr for top round and tenderloin steaks.

Overall desirability scores were higher (P<0.05) for the steaks from the CONV kidney fat removal treatment on day 0 of retail display as compared to ACC steaks, but ACC and CONV steaks had similar overall desirability scores on day 1 of retail

display. Steaks from the ACC kidney fat removal treatment were rated significantly higher ($P<0.05$) for overall desirability on day 2 through day 4 of the retail display period than were the CONV steaks. Higher overall desirability scores for the ACC steaks later in the retail display period possibly could be due to the darker lean color masking some of the surface discoloration present on the ACC steaks.

Steaks from tenderloins removed from the carcass at 6d had significantly higher overall desirability scores on day 1 of retail display than did steaks from tenderloins removed at 2d; otherwise, time of tenderloin removal had only minimal effect on overall desirability scores during the retail display period (Table 4). Steaks from the VAC packaging and storage treatment had higher overall desirability scores on day 0 and 1 but were not significantly different from CONT steaks on day 2 through day 4 of retail display. VAC tenderloin steaks appeared to decline in overall desirability score at a much faster rate than did the CONT tenderloin steaks.

Data from the present study suggested that if kidney, pelvic, and heart fat were allowed to be removed prior to chilling and grading some slight changes in tenderloin quality could be expected. These changes included slightly darker lean color and slightly higher WBS values for tenderloin steaks from sides with KPH fat removed prior to chilling compared to conventional dressing. Since these differences were minor and tenderloin steaks from ACC sides were actually scored higher in overall desirability upon retail evaluation at day 2 through day 4, these findings should not deter packers from removing KPH fat prior to chilling and grading.

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mained practically the same over the temperature range measured.

The dependences found in these model experiments, and their mathematical treatment enable an assessment to be made of the amount of decomposition products that will be formed under different conditions. An example is given in Fig. 14 where the calculated values of the theoretically resulting diketopiperazine were—for the given conditions—compared with the experimental data obtained in a food material, in this case in two new soft drinks sweetened with Usal. The diketopiperazine concentrations determined in the soft drinks stored at 20 ± 2°C were within the theoretical values of diketopiperazine concentrations that would be formed in the model system at the same pH as that of the soft drinks and at the same pH at temperatures between 20°C and 25°C. This might be regarded as in good agreement. The course of diketopiperazine formation in the soft drinks at 7 ± 2°C was similar to that in the model system at 7°C. However, the diketopiperazine concentrations in the soft drinks were higher, due to the higher initial diketopiperazine concentration that was probably caused by diketopiperazine formation during the preparation of the syrup for the soft drinks (different pH and temperature). This fact was not accounted for in the theoretical evaluations. The comparison of the measured and calculated values confirms the validity of the proposed mathematical solution of the amount of Usal decomposition products (especially diketopiperazine) in much more complex systems than those used in model experiments from which they had been derived.

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Effect of Phosphates on the Functional Properties of Restructured Beef Rolls: The Role of pH, Ionic Strength, and Phosphate Type

GRAHAM R. TROUT and GLENN R. SCHMIDT

ABSTRACT

Combinations of sodium chloride and five different phosphates were used to study the effect of phosphate type (chain lengths: 1.0–12.8), ionic strength (0.15–0.43), and pH (5.50–6.35) on the binding ability (cook yield and tensile strength) of restructured beef rolls. The results showed that binding ability increased linearly with increasing ionic strength and pH until a maximum value was reached and then plateaued; approximately 80% of the increase in binding ability was due to the increase in ionic strength and pH. Above an ionic strength of 0.15, the polyphosphates (chain length > 1.0) produced synergistic increases in binding ability with increasing ionic strength. The extent of the synergistic effect decreased linearly as the chain length of the phosphate increased.

INTRODUCTION

BECAUSE OF THE EVIDENCE relating sodium intake to hypertension (Altschut and Grommet, 1980; Pearson and Wolzak, 1982), many health conscious consumers are avoiding foods that contain high sodium levels—particularly processed meat products. As a result, meat processors are now reducing the sodium chloride content of their products (the most practical way of reducing the sodium level). However, one of the consequences of this reduction is a sharp decrease in functional properties (texture, and fat and water binding ability) (Swift and Ellis, 1957; Sofos, 1983). To overcome this problem, meat processors add phosphates to their products since these additives improve the functional properties without appreciably increasing the sodium content (Trout and Schmidt, 1983).

Although most food grade phosphates increase the functional properties of meat products the extent of the increase depends on, amongst other factors, the type of phosphate, the pH of the product, and the concentration of sodium chloride used in the product. As a generalization, the different phosphates increase functional properties in the following order: pyrophosphate > tripolyphosphate > tetrapolyphosphate > hexametaphosphate ≈ orthophosphate (Bendall, 1954; Shults et al., 1972; Trout and Schmidt, 1984). Several studies have shown, however, that phosphates do not increase functional properties when the sodium chloride concentration is below 0.8% (Bendall, 1954; Hellendoorn, 1962), greater than 2.0% (Swift and Ellis, 1957; Trout and Schmidt, 1984), and between 1.25–1.50% if the pH of the product is also high (>6.0–6.3) (Puolanne and Matikkala, 1980; Trout and Schmidt, 1984).

Much of the behavior of phosphates just described has been attributed to differences in ionic strength and pH since both of these parameters affect functional properties (Hamm, 1960; Acton et al., 1982). The differences in effectiveness between phosphates appears to be directly related to the ability of phosphates to increase ionic strength and pH (Trout and Schmidt, 1984). Furthermore, the reason phosphates do not increase functionality at intermediate and high sodium chloride levels is that the combination of high pH and high ionic strength produced by the sodium chloride increase the functional properties to a maximum (Sofos, 1983). Consequently, adding phosphates to meat products under these conditions has no beneficial effect on functionality.

What these two properties do not explain is why at sodium chloride concentrations greater than 0.8%, phosphates increase the functional properties of meat products more than other salts of comparable ionic strength and pH (Bendall, 1954; Hellendoorn, 1962). Two theories have been put forward to explain this behavior, but neither explanation seems completely satisfactory. Bendall (1954) attributed the greater effectiveness of pyrophosphate to its ability to dissociate actomyosin into actin and myosin. But as only pyrophosphate dissociates actomyosin (and possibly tripolyphosphate if it is rapidly hydrolyzed to pyrophosphate in the products [Yasui et al., 1964]), this theory does not explain why other phosphates increase functionality. Hamm (1970) suggested that the reason phosphates increased functionality so effectively is that they bind to the meat proteins. This concept is not completely satisfactory either, since there is no direct relationship between the extent to which phosphates bind to meat proteins and the effect they have on functionality (Trout and Schmidt, 1983).

Research with other food proteins indicates that all salts (and this includes phosphates) have intrinsic properties that increase functional properties (Damodaran and Kinsella, 1982). In contrast to what has traditionally been accepted, this research indicates that the type and molar concentration of salt or phosphate rather than the ionic strength produced by the salt or phosphate determines how the salts affect functionality. This research, however, does not explain why certain salt/phosphate combinations produce synergistic increases in functionality (Bendall, 1954). Nor does it indicate which phosphates produce this synergistic effect or what ionic strength and pH conditions are required for such an effect.

The present study therefore was instituted for two reasons: (a) to determine, at the same ionic strength and pH, the effect of combinations of sodium chloride and five different food grade phosphates on the functional properties (cook yield and tensile strength) of restructured beef rolls, and (b) to determine how ionic strength and pH influence the effectiveness of the phosphates.

MATERIALS & METHODS

Meat

Semimembranosus muscles were removed 48 hr postmortem from one side of twenty 18–24 month old commercially slaughtered steers and trimmed of all visual fat and connective tissue. Samples were taken from three different locations in each muscle for pH determination; the pH of all muscles was between 5.4 and 5.8. Muscles were randomly chosen, ground through a 2.5 cm plate (Hobart Grinder, model 4146), and mixed thoroughly by hand. Ten 100g samples were taken during grinding and pooled for subsequent proximate analysis (AOAC, 1970), hydroxyproline analysis (Stegman and Stadler, 1967), and pH determination. The amount of ground meat required for each treatment (approximately 600g) was weighed into double thickness

Author Schmidt is with the Dept. of Animal Sciences, Colorado State Univ., Fort Collins, CO 80523. Author Trout's present address is Dept. of Animal & Dairy Sciences, Auburn Univ., Auburn, AL 36849.

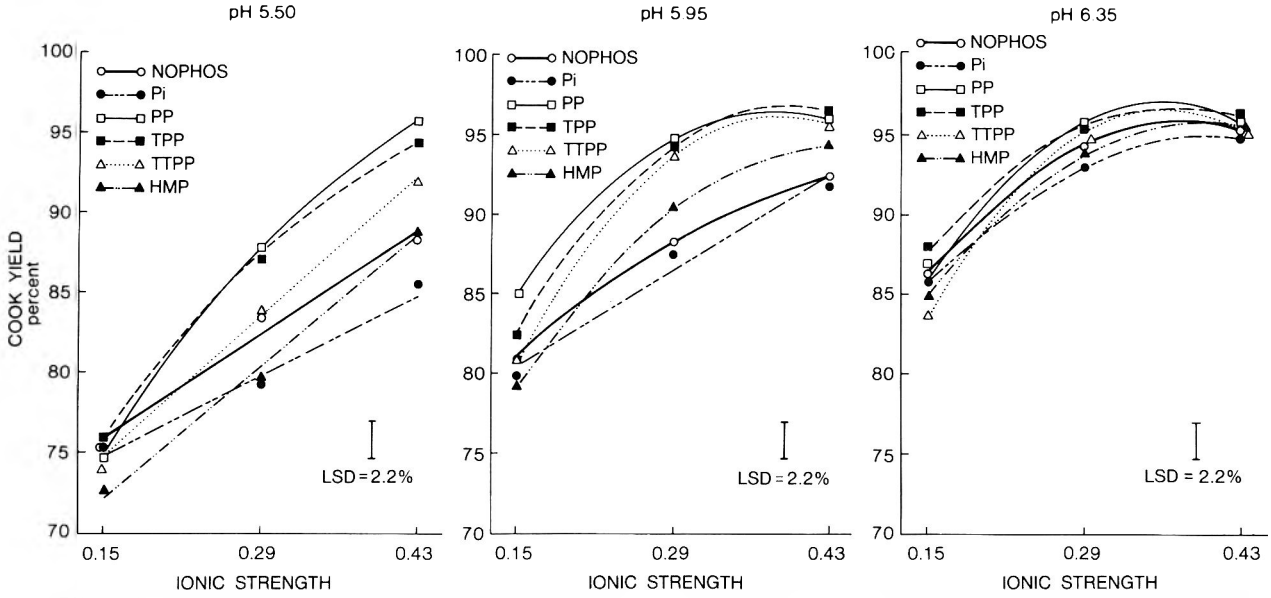

Fig. 1—Effect of ionic strength and type of phosphate on the cook yield of restructured beef rolls at pH values of 5.50, 5.95, and 6.35. The LSD (least significant difference) was calculated at the 5% probability level.

opaque polyethylene bags, sealed, and frozen at −30°C until used (within 6 months). All meat preparation and grinding was carried out in a refrigerated room (8°C).

The meat had the following composition: pH, 5.54; protein, 21.6% (percent N × 6.25); moisture, 74.7%; fat, 1.8%; and collagen, 0.60% (hydroxyproline × 7.25).

Additives

The additives used were sodium chloride, disodium phosphate (Pi) (both analytical reagent grade), deionized water (5% of the produce weight), and the following food grade phosphates: tetrasodium pyrophosphate (PP), sodium tripolyphoshate (TPP), sodium tetrapolyphosphate (TTPP), and sodium hexametaphosphate (HMP) (courtesy of FMC Corporation, Philadelphia, PA). The phosphates were analyzed for number average chain length ($\bar{n}$) using the titration method outlined by Lowenheim (1973). The number average chain length of the phosphates was as follows: PP, 2.0; TPP, 3.0; TTPP, 5.7; and HMP, 12.9. The chain length of Pi was taken to be one. This analysis confirmed that the phosphates had not hydrolyzed during storage and that they were typical of commercially used phosphates (Ellinger, 1972).

Treatments

The treatments investigated (54) were a factorial combination of the six phosphate types (no added phosphate [NOPHOS], Pi, PP, TPP, TTPP, and HMP), the three ionic strength levels (0.15, 0.29, and 0.43), and the three pH levels (5.50, 5.95, and 6.35). The phosphates were used at a constant ionic strength of 0.055 and the different ionic strength levels were obtained by varying the sodium chloride concentration. The phosphate concentrations required to obtain an ionic strength of 0.055 were as follows: Pi, 0.477%; PP, 0.313%; TPP, 0.321%; TTPP, 0.380%; and HMP, 0.420%. The sodium chloride concentrations required to obtain the three ionic strength levels of 0.15, 0.29, and 0.43 were as follows: 0.55%, 1.37%, and 2.19% for the treatments that contained phosphates and 0.88%, 1.70%, and 2.51% for the treatments without phosphates, respectively.

Product pH was controlled by adding predetermined amounts of either 1 M NaOH or HCl to the product during mixing. The acid or base was diluted immediately before addition by mixing it with either the water or the phosphate solution.

The ionic strength of the phosphates was calculated using the molar concentration, chain length, and degree of dissociation as previously reported (Trout and Schmidt, 1986); the ionic strength of NaCl was calculated using the conventional formula:

$$IS = 0.5\Sigma M_i Z_i^2$$

where M_i is the molarity and Z_i is the valency of the species of ion present. The ionic strength contribution of the added NaOH and HCl was taken into consideration when the total ionic strength of the treatments was calculated; this contribution was never more than 1.5% of the total ionic strength.

Processing

Before use, the meat was thawed in 1 liter beakers for 36 hr at 2° to 3°C. To re-incorporate the drip, the meat plus any drip collected in the beaker was transferred to a precooled, preweighed, stainless steel bowl and mixed for 30 sec on speed two with a Kitchen Aid Mixer (Model K45SS Hobart Co., Troy Oh). The rolls were prepared by mixing the meat and ingredients for two min on speed two and then for one min on speed three. Salt, water, and phosphate (when used) were added during the initial stages of mixing; salt was added dry while the phosphates were dissolved in all the water. After mixing, a sample (50g) was taken for pH determination and the mixing temperature was measured; all temperatures were between 0° and 8°C. The meat mixture was stuffed into presoaked, prestuck, fibrous cellulose casings (10 cm flat width) using a hand operated stuffer, and the casings were tensioned and clipped. The rolls were stored for up to 8 hr at 2° to 3°C in polyethylene bags until heat processed. Immediately before heat processing, the rolls were vacuum packaged in moisture impermeable plastic pouches.

The rolls were cooked to an internal temperature of 70°C (typically for 90 to 100 min) in an air agitated, thermostatistically controlled retort as previously described (Trout and Schmidt, 1984).

After heat processing, the rolls were cooled in running tap water (6° to 8°C) to an internal temperature of 35°C (typically 30–35 min), removed from the bags and casings, dried with paper towel, and weighed for cook yield determination. The rolls were stored in polyethylene bags at 2° to 3°C overnight, prior to tensile strength evaluation.

Analysis

pH was determined by blending at high speed in an Osterizer Imperial blender (John Oster Mfg. Co., Milwaukee, WI) 50g of sample with 250 mL deionized water. The pH of the resultant suspension was measured with a Corning Model 125 pH meter equipped with a Corning combination pH electrode (Cat. No. 34107; Corning, Medfield, MA).

Cook yield was calculated from the weight of the paper-towel-dried, cooked roll and the net weight of the roll before heat processing. This value was expressed as a percentage.

Tensile strength was measured on a modified Warner-Bratzler Meat Shear apparatus, (Model 2000, G.R. Electric Mfg. Co., Manhattan, KS) as previously described (Trout and Schmidt, 1984). Tensile strength was calculated from the force required to reach the bioyield point and the cross-sectional area and was expressed as g/cm^2.

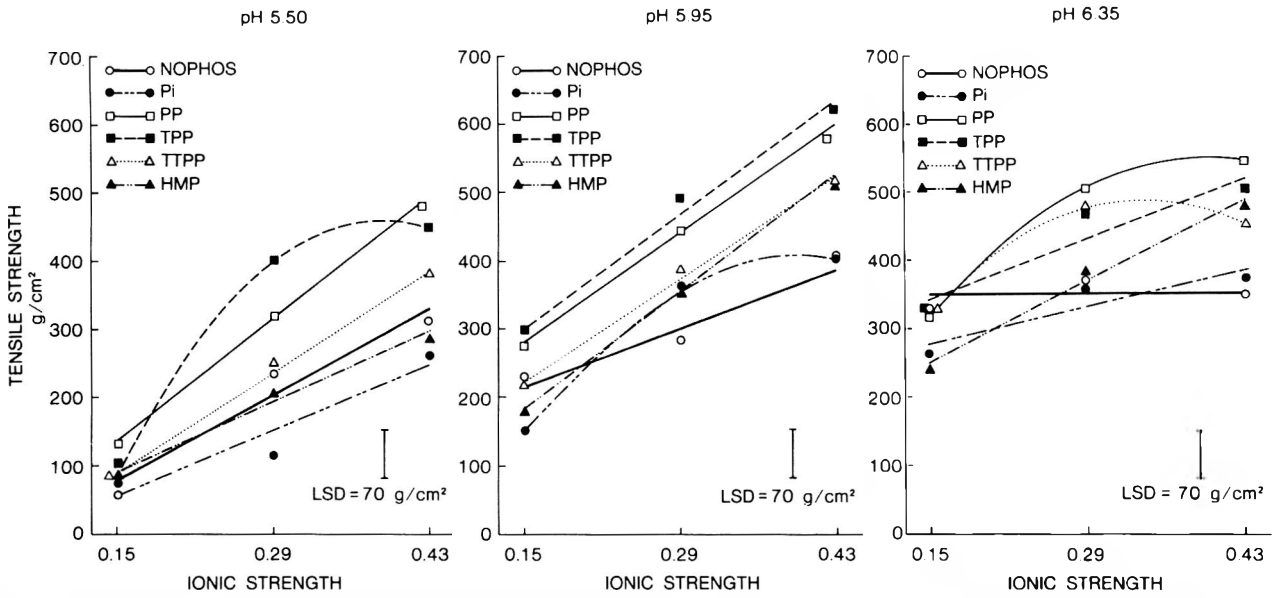

Fig. 2—Effect of ionic strength and type of phosphate on the tensile strength of restructured beef rolls at pH values of 5.50, 5.95, and 6.35. The LSD (least significant difference) was calculated at the 5% probability level.

Experimental design and statistical analysis

The experimental design used was a partially balanced incomplete block design with three replicates. The treatments were arranged as a $6 \times 3 \times 3$ complete factorial (six phosphate types, three ionic strength levels, and three pH levels). Within each replicate, the treatments were assigned to one of three partially balanced incomplete blocks (Bose, 1939) (a total of nine blocks), and all treatments in a block were prepared and cooked on the same day.

The data were analyzed by analysis of variance. The small variation in cook yield and tensile strength due to the deviation of the pH from the predetermined levels was removed by using the uncooked product pH as a covariate in the analysis of variance. Mixing temperature and time from preparation to thermal processing were also included as covariates in the analysis; however, neither variable produced a significant effect ($P<0.20$).

When the analysis of variance showed significant F values, either Fischer's Least Significant Difference (when two means were being compared) or Scheffe's multiple comparison test (when more than two means were being compared) were used to locate differences ($P<0.05$) between treatment means (Snedecor and Cochran, 1976). Significant polynomials ($P < 0.01$) were fitted to the data using the method of Snedecor and Cochran (1976). An all possible subset procedure (SAS, 1979) was used for multiple regression analysis. The regression equations obtained were subjected to case analysis to check the adequacy of the fitted model (Weisberg, 1980).

RESULTS

Effect of ionic strength

Increasing the ionic strength of the restructured rolls increased both the cook yield (CY) (Fig. 1) and tensile strength (TS) (Fig. 2). At the lower pH levels, CY and TS increased linearly with increasing ionic strength ($P<0.01$). At the higher pH levels, CY and TS increased either quadratically ($P<0.01$) or did not increase significantly ($P>0.05$). These non-linear trends occurred when CY and TS were maximum at one or more ionic strength level.

The rate at which CY and TS increased with increasing ionic strength depended on both the pH of the product and the type of phosphate and was greatest when the pH was low (< 5.95) and when PP, TPP, and TTPP were used. CY and TS increased rapidly at low pH most probably because the CY and TS values were low and hence large increases in CY and TS were possible. However, the rapid increase in CY and TS that was observed with the PP, TPP, and TTPP treatments occurred because these phosphates increased in effectiveness more rapidly with increasing ionic strength than the other phosphates.

The most important effect of ionic strength in this study was to increase the phosphates' ability to increase CY and TS. At low ionic strength (0.15), the phosphates increased CY and TS to the same extent as the NOPHOS treatment ($P>0.05$). However, as the ionic strength increased above 0.15, the phosphates became progressively more effective at increasing CY and TS (relative to the NOPHOS treatment); i.e. at the higher ionic strengths the phosphates produced synergistic increases in CY and TS. Under most conditions, the rate at which CY and TS increased with increasing ionic strength (as measured by the slope of the line or the initial slope of the nonlinear curves) was greatest for the shorter chain length phosphates and decreased as the chain length of the phosphate increased. Pi, the shortest chain phosphate, did not increase in effectiveness with increasing ionic strength and had a similar effect on CY and TS as the NOPHOS treatment.

Because of the interactions between the ionic strength and the type of phosphate, the phosphates did not increase functionality to the same extent over the entire range of ionic strength and pH conditions studied. Hence, the phosphates can not be ranked exactly, based on their effectiveness under all conditions. In general terms, though, their effectiveness was as follows: PP, TPP, and TTPP were always as effective as, or more effective than, NOPHOS; HMP was either as effective as, or slightly more effective than, NOPHOS; and Pi was either as effective as, or slightly less effective than, NOPHOS.

At pH 6.35, ionic strength did not have same effect on CY and TS as it did at the lower pH levels.

With CY, it appears that at pH 6.35 increasing the ionic strength did not increase the effectiveness of the phosphates (Fig. 1), as there was no significant difference in CY between NOPHOS and any of the phosphate treatments, at any of the three ionic strength levels. This effect was most likely due to the conditions used rather than the pH *per se*. At pH 6.35, none of the phosphates increased CY more than NOPHOS at low ionic (0.15), which is consistent with what occurred at the lower pH levels. At ionic strengths greater than 0.15, where it was expected that some of the phosphates would be more effective than NOPHOS, CY was maximum for all treatments. Hence, the conditions were such that the phosphates could not increase functionality more than NOPHOS.

The other anomaly at pH 6.35 was that with NOPHOS and

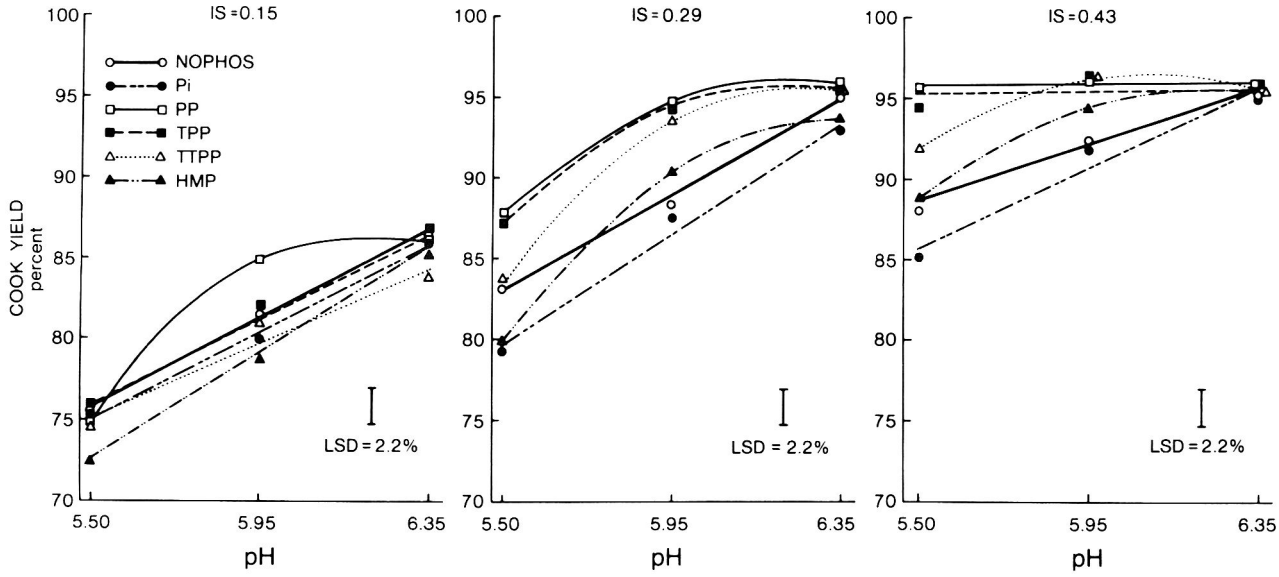

Fig. 3—Effect of pH and type of phosphate on the cook yield of restructured beef rolls at ionic strengths of 0.15, 0.29, and 0.43. The LSD (least significant difference) was calculated at the 5% probability level.

Pi, TS increase very little as the ionic strength increased (Fig. 2); NOPHOS produced a nonsignificant ($P>0.05$) increase, and Pi produced an increase which was approximately half of that produced by the other phosphates.

Effect of pH

To determine how CY and TS changed with increasing pH, the data were replotted within each ionic strength level using pH as the independent variable. Polynomials were fitted to the data to determine the significance of the trends.

The replotting (Fig. 3 and 4) showed that under most conditions, CY and TS increased linearly ($P<0.01$) with increasing ionic strength. At the higher ionic strength levels, CY and TS were at a maximum at one or more pH level for some of the treatments. In these cases, the increase was either quadratic ($P<0.01$) or there was no significant increase ($P>0.05$). CY and TS increased most rapidly when the other treatments were least effective; i.e, when the ionic strength was low and when HMP, Pi, or NOPHOS were used.

At the highest ionic strength (0.43), pH had little effect on either CY or TS. This was particularly true with TS; all phosphate produced maximum TS at pH 5.95. An effect similar to this has previously been reported with heated myosin gels; in 0.6M KCl, myosin produces maximum gel strength at pH 6.0 (Ishioroshi et al., 1980).

Unlike ionic strength, pH did not enhance the phosphates ability to increase CY and TS but seemed to have the same effect on the phosphate treatments as it did on the NOPHOS treatment. At low ionic strength (0.15), this general effect of pH was very apparent as the rate at which CY and TS increased with increasing pH (i.e., the slopes of the curves) was the same ($P>0.05$) for NOPHOS and all phosphate treatments. At higher ionic strengths, however, this general effect of pH was not so apparent as the rate at which CY and TS increased with increasing pH varied considerably ($P>0.05$) between treatments.

This variation in rate of increase did not appear to be due to an interaction between pH and the phosphates but appeared to be the result of the previously discussed interaction between

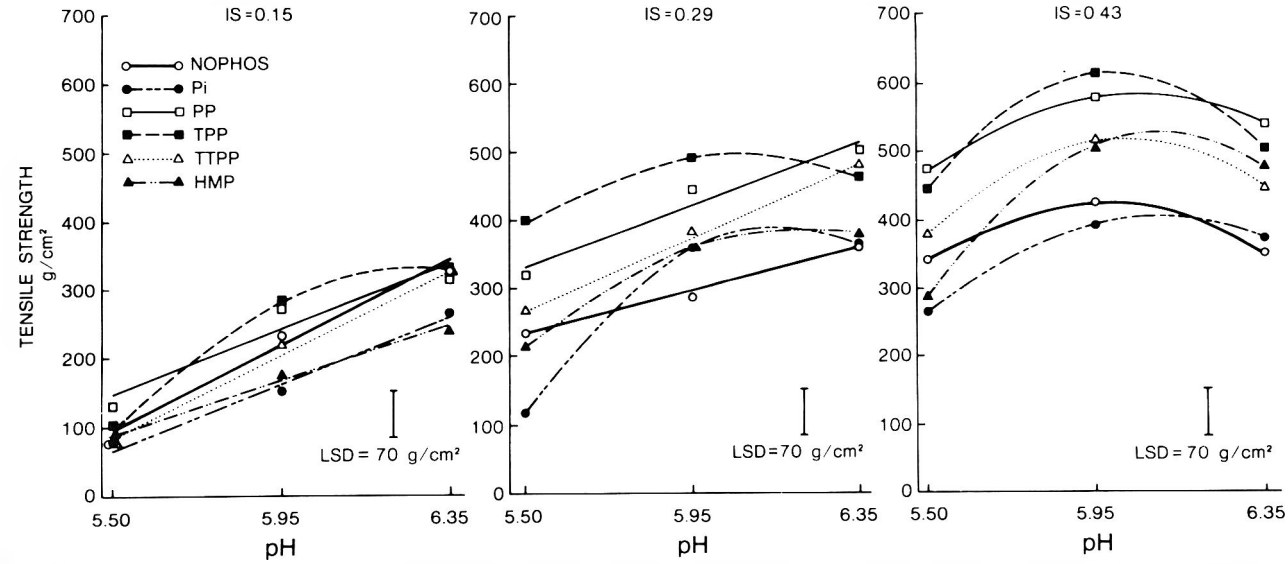

Fig. 4—Effect of pH and type of phosphate on the tensile strength of restructured beef rolls at ionic strengths of 0.15, 0.29, and 0.43. The LSD (least significant difference) was calculated at the 5% probability level.

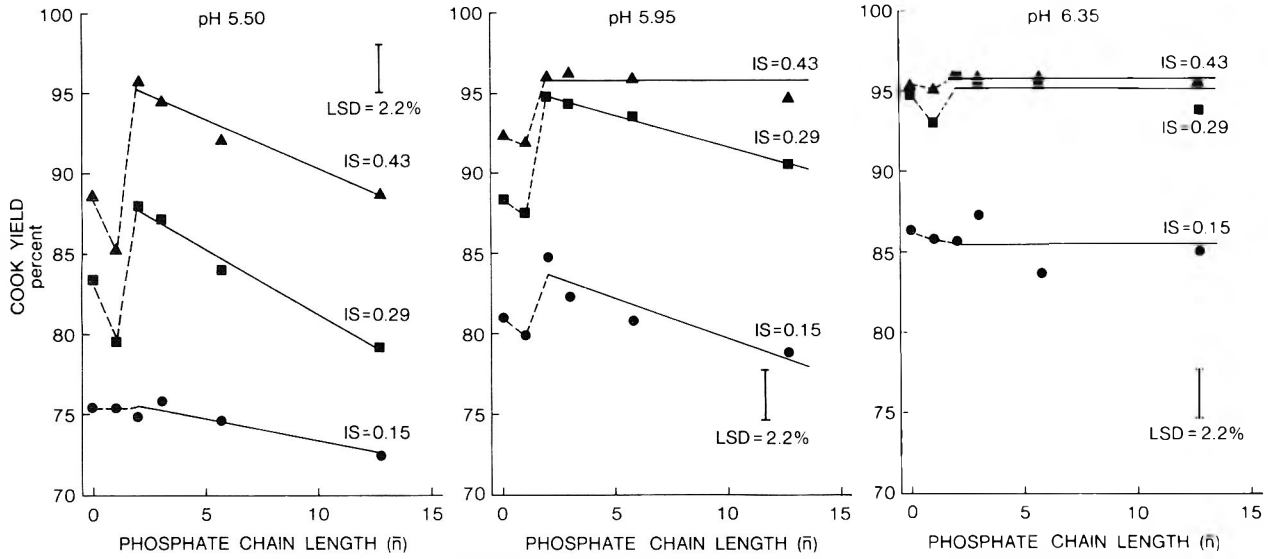

Fig. 5—Effect of phosphate chain length on the cook yield of restructured beef rolls at pH 5.50, 5.95, and 6.35 and ionic strengths of 0.15, 0.29, and 0.43. The treatments with a phosphate chain length of zero contain no phosphate, only sodium chloride. The LSD (least significant difference) was calculated at the 5% probability level.

ionic strength and the phosphates. This behavior can be explained in the following manner. At low ionic strength (0.15) all phosphates were equally effective and their rates of increase were the same. At ionic strengths greater than 0.15, the CY and TS values of the phosphates most affected by ionic strength, i.e, PP and TPP, were higher at pH 5.50 than the corresponding CY and TS values of the other phosphate treatments. Hence, as the CY and TS values for these phosphates were high at low pH, the values increased at a lower rate as the pH increased.

What further indicates that there is no direct interaction between pH and the effectiveness of the phosphates is that at all three ionic strength levels, the initial rate of increase of CY and TS with increasing pH (i.e., the slopes of the linear curves or the initial slopes of the curvilinear curves) was very similar for NOPHOS and all phosphate types. Furthermore, earlier work has shown that, in the same pH range investigated here, pH had little direct effect on the ability of either PP or TPP to increase the water binding ability of model meat products (Hellendoorn, 1962).

Effect of phosphate chain length

From the data it appeared that if the effect of Pi was excluded, the effectiveness of the phosphates decreased as the chain length of the phosphate increased. To determine if this was a systematic effect, the CY and TS results were replotted using phosphate chain length as the independent variable. Polynomials were fitted to the data (excluding the NOPHOS and Pi treatments) to determine the significance of the trends. This analysis was carried out within each of the nine ionic strength-pH combinations investigated.

The trend analysis of the data (Fig. 5 and 6) showed that in most cases the ability of the phosphates to increase CY and TS decreased linearly ($P<0.05$) as the chain length of the phosphate increased. However, phosphate chain length had no significant effect on CY and TS ($P>0.05$) at the higher ionic strength-pH combinations if one or more phosphate had produced maximum CY or TS.

The rate at which CY and TS decreased per unit increase in phosphate chain length ranged between 0.4% and 0.8% for CY, and 7.6 g/cm^2 and 13.4 g/cm^2 for TS. As a generalization, the rate of decrease was lowest at the low ionic strength and increased (i.e., the rate became more negative) as the ionic strength increased. The reason the slope increased with increasing ionic strength was that the shorter chain length phosphates increased in effectiveness more rapidly with increasing ionic strength than the longer chain phosphates and not because the longer chain phosphates decreased in effectiveness with increasing ionic strength. (Fig. 5 and 6).

Effect of other phosphate properties

Results from an earlier study (Trout and Schmidt, 1984) indicated that regardless of the type of salt or phosphate, maximum CY and TS should occur when both the ionic strength and pH of the product were greater than 0.34 and 6.0 respectively. In this current study, PP, TPP, and TTPP produced maximum CY under these conditions, but none of the treatments produced maximum TS. However, NOPHOS and all phosphate treatments produced maximum CY and TS when the ionic strength was between 0.29 and 0.43 and the pH was between 5.95 and 6.35. Although NOPHOS and Pi produced maximum TS under the same conditions as the other phosphates, the maximum TS value was considerably lower (400g/cm^2) than the average maximum value produced by the other phosphates (550g/cm^2).

The percentage of the increase in CY and TS that could be attributed to (a) the increase in ionic strength, (b) the increase in pH, and (c) the presence of phosphate was calculated from the analysis of variance by dividing the sum of squares for each variable by the total sum of squares. Because of the interactions between variables, only a range of values was calculated for each variable; the minimum and maximum values in this range were calculated from the sum of squares and the sum of squares plus interaction sum of squares, respectively. The percentage of the variation in CY explained by each variable was as follows: ionic strength, 53.5–59.5%; pH, 24.7–30.5%; and presence of phosphate, 4.7–8.9%. The corresponding percentages for TS were: ionic strength, 45.3–53.6%; pH, 21.8–28.4%; and presence of phosphate, 12.7%–18.9%.

Although most of the variation in CY and TS could be attributed to the variation in ionic strength and pH (80–95%), an appreciable amount of the variation, particularly the variation in TS, was due to some other property of the phosphates. This is in contrast to the results of an earlier study (Trout and Schmidt, 1984) where, due to the possible confounding of the effect of the phosphate ion with the effect of pH, it was concluded that phosphates increase functionality mainly by increasing ionic strength and pH.

These results, however, do not indicate how important this

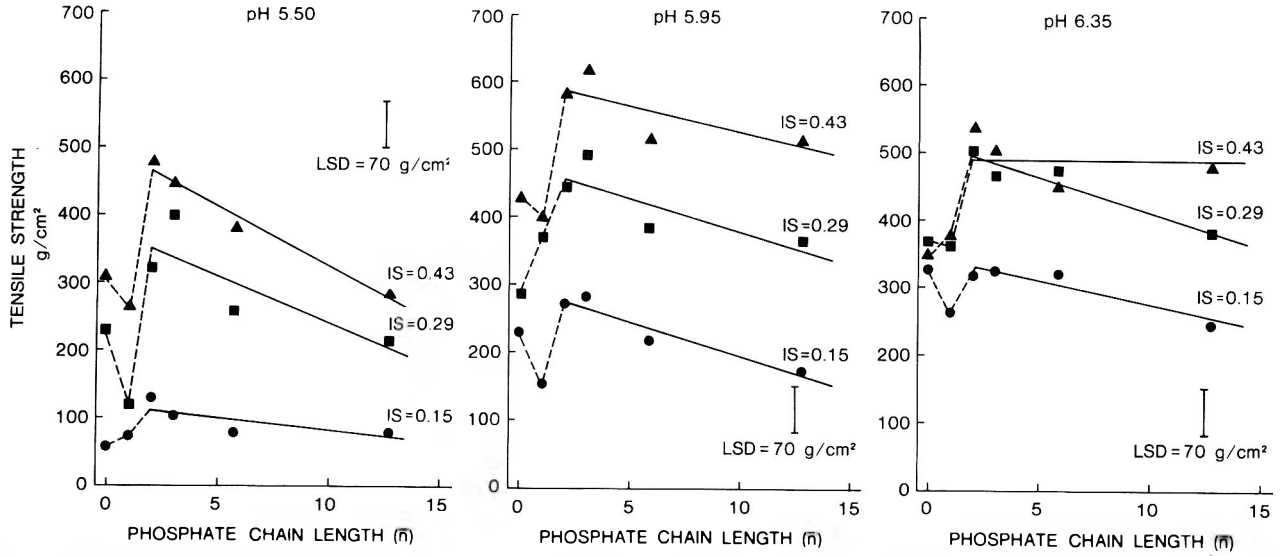

Fig. 6—Effect of phosphate chain length on the tensile strength of restructured beef rolls at pH 5.50, 5.95, and 6.35 and ionic strengths of 0.15, 0.29, and 0.43. The treatments with a phosphate chain length of zero contain no phosphate, only sodium chloride. The LSD (least significant difference) was calculated at the 5% probability level.

intrinsic phosphate property is when the phosphates are used in meat products without pH adjustment. To obtain this information, mulitple regression equations were developed for NO-PHOS and each of the five different phosphates that predict CY and TS based on the ionic strength and pH of the product. Since the ionic strength and pH increases produced by each phosphate were known (the ionic strength increase was predetermined and the pH increases were calculated from the pH adjustment data), the regression equations could be used to determine the increase in CY and TS produced when the phosphates were added to products of given sodium chloride concentration and initial product pH. Using these equations the increase in CY or TS attributable to the intrinsic phosphate property was determined by subtracting from the CY or TS value obtained with the phosphate the corresponding value obtained when the ionic strength and pH were increased to the same extent with sodium chloride and sodium hydroxide, respectively.

The results in Table 1 show the percentage of the increase in CY and TS attributable to this intrinsic phosphate property for PP and HMP (the shortest and longest chain length polyphosphates, respectively), at three sodium chloride concentrations, and two initial pH levels. When the differences in ionic strength and pH are taken into consideration, PP was almost twice as effective at increasing functionality as HMP even though the weight concentration of PP was 35% less than that of HMP. Furthermore, the effectiveness of both phosphates increased as the salt concentration increased, but their effectiveness increased only slightly and non-systematically as the pH increased. The results also show that both phosphates had a greater effect on TS than on CY, particularly when the sodium chloride concentration and pH were both high (greater than 1.50% and 6.0, respectively).

DISCUSSION

OUR RESULTS INDICATE that ionic strength, pH, and the addition of phosphates increase the functional properties of meat products (i.e., CY and TS) by a similar mechanism since each variable had the greatest effect on the functional properties when the other variables were least effective, and *visa versa*. In a series of experiments, Puolanne and Ruusunen (1980) and Puolanne and Matikkala (1980) found that in frankfurters, sodium chloride, pH, and PP had a similar effect on functionality as described here.

Our results also indicate that phosphates do not increase functionality solely by increasing ionic strength and pH. To illustrate this point, at ionic strengths greater than 0.15, phosphates produce synergistic increases in functional properties when used with sodium chloride. Previously, it was shown that PP produced a similar synergistic effect at ionic strengths greater than 0.14 (Hellendoorn, 1954). Furthermore, phos-

Table 1—Increase in cook yield and tensile strength of restructured beef rolls produced by the addition of pyrophosphate and hexametaphosphate (both with an ionic strength of 0.055) and percentage of this increase due to intrinsic phosphate properties (i.e., the percentage not due to the increase in ionic strength and pH), at various sodium chloride concentrations and pH levels

			Cook yield (%)			Tensile strength (g/cm²)		
pH[a]	Phosphate type	NaCl (%)	Without phosphate	With[b] phosphate	Percent[c] increase due to phosphate	Without phosphate	With[b] phosphate	Percent[c] increase due to phosphate
5.50	PP	0.8	73.4	84.7	16.5	47	304	32.3
		1.5	80.8	92.8	25.4	179	474	45.8
		2.2	87.0	96.8	43.0	258	586	57.1
	HMP	0.8	73.4	81.2	7.6	47	207	16.6
		1.5	80.8	88.5	12.7	179	341	26.7
		2.2	87.0	92.9	23.0	258	419	37.2
6.00	PP	0.8	80.9	90.3	19.7	198	385	44.6
		1.5	90.1	96.0	25.3	305	508	63.0
		2.2	93.8	96.0	48.0	325	563	78.6
	HMP	0.8	80.9	88.2	8.2	198	320	21.9
		1.5	90.1	94.9	20.5	305	427	35.6
		2.2	93.8	95.0	31.5	325	469	41.7

[a] pH before the addition of phosphates; the pH increases due to the addition of PP and HMP were 0.33 and 0.17, respectively.
[b] Increase in cook yield or tensile strength due to the addition of PP and HMP of ionic strength 0.055 (see text for weight concentrations).
[c] Percentage of the increase in cook yield and tensile strength not attributable to the increase in ionic strength or pH.

phates increase TS at high ionic strength and pH (greater than 0.29 and 6.00, respectively), conditions under which TS can not be increased by increasing the pH or by increasing the ionic strength with sodium chloride (Fig. 6).

Mode of action of phosphates

Although the changes in functional properties produced by phosphates can not be explained in terms of changes in ionic strength and pH, these changes seem consistent with current knowledge of how salts affect the denaturation behavior and hence functional properties of food proteins.

It is generally accepted that salts improve the functional properties of meat products (a) by facilitating the extraction of the structural myofibrillar proteins from the muscle cells during mechanically treatment (i.e., mixing, chopping, tumbling, etc), and (b) by interacting with the muscle proteins during heating so that the proteins form a strong matrix that entraps free water and gives the products its texture (Acton et al., 1983; Schmidt et al., 1981). Extraction of myofibrillar proteins, however, appears to have only a small effect on functional properties. This conclusion is based on the following observations: (1) Under many conditions, myofibrillar protein extraction and meat protein functionality are poorly related (Knipe et al., 1985); (2) extracted myofibrillar proteins have poor functional properties when heated in the absence of salts and/or phosphates (Macfarlane et al., 1977; Siegel et al., 1978). Hence, this discussion will center around the thermally induced changes in the meat protein matrix.

Most salt induced increases in protein functionality are the result of changes in protein conformation (Damodaran and Kinsella, 1982). Because of these conformational changes, the proteins form a characteristic three dimensional lattice structure when heated. This lattice structure is the molecular basis for the heat-set protein matrix previously described. Salts produce these conformational changes by altering the hydrophobic and electrostatic interactions that stabilize the protein structure (Franks and Eagland, 1975). At high salt concentrations salts primarily affect the hydrophobic interactions, since salts have little effect on electrostatic interactions at ionic strengths greater than 0.1 (Melander and Horvath, 1977). Above an ionic strength of 0.1, the high concentration of ions surrounding the charged protein residues shield the residues, preventing them from interacting with other charged particles (Von Hippel and Schleich, 1969).

The ionic strength of all treatments in this study were greater than 0.1 (all were between 0.15 and 0.43). Therefore, it is highly unlikely that the electrostatic properties of the phosphates had any effect on the functional properties of the meat proteins. Furthermore, since ionic strength is a measure of the electrostatic properties of the phosphates, it is understandable that, at constant pH, the behavior of the phosphates can not be explained solely in terms of changes in ionic strength.

How functional properties change with changes in salt type and concentration give a good indication as to which interactions salts are affecting, since hydrophogic and electrostatic interactions are affected differently by such changes. Electrostatic interactions are affected by the charge on the ions; hence, different salts with the same charge and at the same concentration (i.e., at the same ionic strength) have the same effect on these interactions. The extent of this effect increased nonlinearly with increasing concentration (Melander and Horvath, 1977). In contrast, different salts at the same concentration have different effect on hydrophobic interactions, and the extent of the effect increases approximately linearly with increasing molar concentration (Melander and Horvath, 1977).

Two major results in this study indicate that phosphates increase muscle protein functionality mainly by altering hydrophobic interactions.

The first result was the linear decrease in effectiveness of the phosphates with increasing chain length. These changes in functionality must have been due, at least in part, to changes in hydrophobic interactions. If they were due to changes in electrostatic interactions, all treatments at the same ionic strength and pH (including the sodium chloride treatment) would have increased functionality to the same extent. The fact that the effectiveness of the phosphates decreased linearly with increasing chain length may have been due to the increase in phosphate chain length *per se* (i.e., a change in type of phosphate). It is also possible that this linear decrease was due to a decrease in molar concentration. Although the weight concentrations of all phosphates were similar, because the molecular weight increase with increasing phosphate chain length, the molar concentration decreases accordingly. Regression analysis of the data showed that the decrease in functionality was linearly related ($P<0.01$) to the decrease in molar phosphate concentration. Of course, the linear decrease in effectiveness may have been due to the combined effect of increased chain length and decreased molar concentration.

The second result was the lack of an effect of pH on the effectiveness of the phosphates. If the phosphates were altering electrostatic interactions, altering the pH should increase the phosphates effectiveness. Altering the pH would alter the charge on the phosphates and on the meat proteins which would increase the electrostatic interactions between the two. This would, in turn, alter the conformation of the proteins and hence their functional properties. Since pH had no obvious effect on the behavior of the phosphates, this indicates that the hydrophobic effects of the phosphates had the predominant effect on functionality.

Furthermore, the synergistic increase in functionality produced by the phosphates at ionic strengths greater than 0.15 can be explained in terms of opposing effects of electrostatic and hydrophobic interactions. At low ionic strength, phosphates alter protein conformation mainly by changing the electrostatic interactions (Melander and Horvath, 1977). With meat proteins, it appears that these changes in protein conformation reduce the functional properties of the proteins (Trout, 1984). As the ionic strength increases the electrostatic effects of the phosphates decrease. This occurs partly because of increased ion shielding (as previously discussed) and partly because of the decreased charge on the phosphates due to the increased sodium sodium ion concentration associated with the higher ionic strength (Glasstone and Lewis, 1970). Once the electrostatic effects of the phosphates are reduced, the hydrophobic effects of the phosphates become dominant. And, presumably, it these changes in hydrophobic interactions that increase the functional properties.

If, as these results indicate, changes in hydrophobic interactions are mainly responsible for salt induced changes in meat protein functionality, then knowing the type and molar concentration of salt is more important in determining the effects the salts will have on functional properties than knowing the ionic strength produced by the salt. If this is the case, it should be possible to accurately predict the CY and TS of meat products based on their pH and molar sodium chloride and sodium phosphate concentration. Results from multiple regression equations fitted to the data (not including the Pi data) support this concept. They show that most of the variation in CY (92.6%) and TS (86.0%) can be explained in terms of these three variables.

Dissociation of actomyosin

Results from this study indicate that phosphates other than those that dissociate actomyosin are effective at increasing the functional properties of meat products. In contrast, Bendall (1954) theorized that the effectiveness of phosphates is related to their ability to dissociate actomyosin. In a recent study, Morita and his colleages (1983) found that PP is virtually completely hydrolyzed during manufacture and the initial stages of thermal processing of meat products. They concluded that their

data supports Bendall's hypothesis, since PP hydrolysis is an indication of actomyosin dissociation.

It is doubtful, however, if PP is completely hydrolyzed in meat products. If completely hydrolyzed, PP would not increase functionality. First, complete hydrolysis of PP would indicate actomyosin is not dissociated, since PP must be present for actomyosin to remain in the undissociated form (Granicher and Portzehl, 1964). Furthermore, hydrolysis of PP produces Pi, which this study and an earlier study by Bendall (1954) has shown has little beneficial effect on functionality. In addition, other researchers using more direct techiques have found that phosphates are not extensively hydrolyzed in meat products (Covello and Scheltino, 1965; Molins et al., 1985).

A possible explanation for the results obtained by Morita et al. (1983) is that although their method accurately measures PP concentration, it does not measure PP hydrolysis, only its disappearance. The decrease in PP concentration could have occurred because the PP bound to the meat proteins (PP binds to many proteins [Naus et al., 1969; Vandegrift and Evans, 1981]) and hence could not be detected with their method.

CONCLUSION

INCREASING the ionic strength (from 0.15 to 0.43) and pH (from 5.50 to 6.35) of precooked beef rolls produced predictable, additive increases in CY and TS. Both functional properties increased linearly with increasing ionic strength and pH until the maximum values was reached and then plateau. These maximum values occurred when the pH and ionic strength was between 5.95 and 6.35 and 0.29 and 0.43, respectively (i.e., at sodium chloride concentrations between 1.7%-2.5% and 1.4%-2.2% in the absence and presence of phosphates, respectively). Most of the increase in CY and TS in this study was due to the increase in ionic strength and pH (approximately 53% and 26%, respectively).

Phosphates had a complex effect on CY and TS. With the exception of orthophosphate, all phosphates produced a synergistic increase in CY and TS at ionic strengths greater than 0.15 (i.e., above a sodium chloride concentration of 0.7%), and the extent of this effect increased with increasing ionic strength. The ability of phosphates to produce a synergistic effect decreased as their chain length increased. The phosphates did not produce a similar synergistic effect when the pH was increased. Additionally, the phosphates increased TS at high ionic strength and pH, conditions under which TS could not be increased by increasing the pH or by increasing the ionic strength with sodium chloride.

The effectiveness of the phosphates was determined by the type and molar concentration rather than the ionic strength produced by the phosphate. When compared on a molar basis and at the same ionic strength and pH, all polyphosphates had a similar effect on functional properties; whereas, Pi had a much smaller effect.

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Effects of Chloride Salts on Appearance, Palatability, and Storage Traits of Flaked and Formed Beef Bullock Restructured Steaks

M. F. MILLER, G. W. DAVIS, S. C. SEIDEMAN, and C. B. RAMSEY

ABSTRACT

Restructured steaks made with 0.5 or 1.0% KCl, 0.5% $MgCl_2$ and 0.5% $CaCl_2$ were more desirable and darker red in raw color than blends formulated with 0.5 or 1.0% NaCl. Visual properties of raw steaks containing 0.5 or 1.0% chloride salt were scored higher than the control (no salt added) in 14 of 16 orthogonal contrast mean comparisons. Steaks made with 1.0% $CaCl_2$ or $MgCl_2$ were rated lower than the control in flavor desirability and overall satisfaction ratings. Control, 0.5 or 1.0% NaCl or 0.5 and 1.0% KCl steaks were not different in juiciness, tenderness, flavor desirability or overall satisfaction ratings. Steaks made with chloride salts were rancid after 70 days frozen storage. Results showed steaks made with KCl were superior to steaks formulated with $CaCl_2$ or $MgCl_2$.

INTRODUCTION

THE FUNCTIONS of sodium chloride in processed meats have been established (Hamm, 1960; Theno et al., 1978). However, sodium-related hypertension has become a major national health concern because it may increase the occurrence of symptoms associated with coronary heart disease and stroke (Jacobson and Liebman, 1981). Dietary intakes of sodium may range from 3,900–4,700 mg (10–12g of NaCl) per person per day which is 20–25% times greater than the minimum adult requirement (IFT, 1980), and processed foods may contain 40–60% of total sodium intake Recommendations to reduce the excessive intake of sodium have been made by AMA (1978), FMI (1981), and USDA (1980a, b). They also suggest that processed meats be eliminated or severely reduced in American diets. The need for meat products with reduced amounts of sodium has become more evident since 20% of the American population has been shown to have hypertension (Tobian, 1979; Pearson and Tauber, 1984). The effects of sodium chloride and/or other chloride salts on the safety, sensory or textural properties of frankfurters (Hand et al., 1982b), pork sausage (Terrell et al., 1983), bologna (Lusby and Olson, 1982) and cured sausages (Sofos, 1982) have been studied and show that they improve the desirability of textural properties and emulsion stability of processed meats (Puolanne and Terrell, 1983). However, freezer storage life, textural properties and visual characteristics have not been documented and need to be studied for restructured beef steaks formulated with salt substitutes. Potassium chloride, magnesium chloride and calcium chloride may be possible alternatives to sodium chloride in restructured beef steaks. The use of these salts as replacements for NaCl has not been previously studied in restructured beef steaks and may be of use in extracting meat proteins to improve the binding of meat particles in restructured beef steaks (Seideman and Durland, 1983). The purpose of this study was to examine the storage life, binding, sensory and visual traits of restructured beef steaks formulated with added NaCl or salt substitutes KCl, $MgCl_2$ and $CaCl_2$.

Authors Davis and Ramsey are with the Meats Lab., Animal Science Dept., College of Agricultural Sciences, Texas Tech Univ., Lubbock, TX 79409. Author Miller's present address is Kleberg Center, Meats & Muscle Biology Section, Dept. of Animal Science, Texas A&M Univ., College Station, TX 77843. Author Seideman is with the Roman L. Hruska, U.S. Meat Animal Research Center, ARS, P.O. Box 166, Clay Center, NE 68933. Address inquiries to M.F. Miller.

MATERIALS & METHODS

NINE BEEF BLENDS (two replications, 18 blends, 9.1 kg raw meat) were formulated into restructured beef steaks at the Roman L. Hruska U.S. Meat Animal Research Center (Clay Center, NE). Beef lean was obtained from the rounds and chucks of bull carcasses and frozen at −35°C. The beef lean was segmented into strips (3 × 3 × 30 cm) on a band saw, tempered to −5°C and flaked using an Urschel Comitrol (Model 3600). Flaked and formed mixtures were mixed in a paddle mixer for 20 min with additions of 0.25% sodium tripolyphosphate (STP) and 0.5% or 1.0% of NaCl, KCl, $MgCl_2$ or $CaCl_2$. The salts and phosphate used were 99% pure reagent grade chemicals purchased from Fisher Scientific Company. All blends were stuffed into 16-cm cellulose casings and frozen to an internal temperature of −35°C. The meat logs were tempered to −5°C, pressed at 28 kg/cm^2 in a Ross Superform Press (Model 720), refrozen and cut into 2.5 cm steaks.

Three representative steaks from each formulation were tempered to about −3°C before being cooked to an internal temperature of 70°C on Farberware Open Hearth electric broilers. Internal temperature was monitored by Copper/Iron thermocouple probes and printed on a Leeds and Northrup Speedomax Recorder; cooking time required to reach 70°C was recorded. Steaks were weighed frozen, thawed and again after cooking to determine thaw and cooking losses. After cooking, each steak was cut and visually scored for internal degree of doneness (6 = very rare, 1 = very well done) using photographic standards supplied by the National Live Stock and Meat Board. One cm square samples of the steaks were served to an eight-member sensory attribute panel trained according to Cross et al. (1978). Each member independently evaluated each sample for juiciness, tenderness, bite, cohesiveness, flavor desirability, overall satisfaction and defect description with 8 = extremely juicy, tender, firm, cohesive, desirable, and 1 = extremely dry, tough, soft, crumbly, undesirable. Restructured steaks were scored for defects connective tissue, chunkiness, crumbly, coarse, mushy or off-flavor.

Three representative steaks from each treatment were objectively measured for color using a D-25 Hunter Colorimeter after thawing for 24 hr at 3°C. A five-member trained visual panel evaluated each blend for raw lean color (8 = extremely cherry red; 1 = extremely dark brown) and overall desirability (8 = extremely desirable; 1 = extremely undesirable) according to Jeremiah and Greer (1982). Cooked lean color (8 = extremely light brown; 1 = extremely dark brown) and overall acceptability (8 = extremely desirable; 1 = extremely undesirable) also were evaluated. Steaks were displayed in a Tyler (Model DGC6) retail case under Sylvania Gro-Lux F4040W fluorescent lighting at 80 foot candles following commercial lighting conditions.

The blends were analyzed for moisture, fat and pH. Moisture was determined using the vacuum oven drying procedure; fat was determined by ether extraction (AOAC, 1980). Protein and ash were determined using the procedure described by AOAC (1980). Thiobarbituric acid (TBA) analysis (Tarladgis et al., 1960) for the measurement of rancidity in lipids was conducted on samples stored at −20°C for 10, 50, 70, and 90 days. Three steaks were cooked as described above for sensory evaluation and sampled for textural properties. After a 2-hr cooling period, 1 × 1 × 1 cm diameter cubes were removed from each steak. The textural variables of the test samples were measured by the Instron Universal Testing Machine (Model 1132)/Microcon II using the Warner Bratzler load cell. The fail criterion was set at 75%, the chart paper speed at 20 cm/min and the crosshead speed at 5 cm/min. Six measurements were taken (peak energy, peak load, peak

elongation, fail energy, fail load and fail elongation) on six 1 × 1 × 1 cm cubes per sample. Because the data analysis revealed few differences among the six textural measurements, only the peak load, fail energy and fail elongation data will be presented.

An analysis of variance (Steel and Torrie, 1980) for a 9 (blend) × 3 (level) × 2 (replication) factorial design and appropriate interactions was used to analyze all traits. Seven orthogonal contrasts were made with treatment means. Contrasts 1, 2, 3 and 4 are comparisons of the control to each kind of salt additive. Contrasts 5, 6 and 7 were comparisons of the effectiveness of NaCl to each of three salt substitutes KCl, $MgCl_2$ and $CaCl_2$. The predetermined acceptable level of probability was 5% for all analyses and was used throughout this discussion.

RESULTS & DISCUSSION

DATA not shown in tabular form indicate percentage fat, moisture, thaw loss, cooking loss, doneness score, pH and cooking time were not affected by salt level or salt type. These data were not unexpected since the blends were formulated to contain the same fat content and were cooked to the same endpoint temperature and doneness. Also the steaks were thawed in a similar manner and precut to the same thickness prior to cooking. The levels of salt used in this experiment did not increase the pH above the control. The addition of the STP to the control blend may have caused a high enough increase in pH to equal the salt added blends. Additionally the buffering capacity of the meat may allow little difference to be shown.

Contrast means for visual traits (Table 1) indicated control restructured steaks were rated lower for raw color and raw desirability than steaks which were formulated with the four salts in 14 of 16 comparisons (C1, C2, C3 and C4). These data agree with Huffman et al. (1981) who also reported improvements in raw color with increased salt levels. Hunter A values for C1, C2, C3 and C4 show that increasing salt levels to 1.0% formed and maintained a darker red color in raw restructured steaks. The darker red color would explain why the raw color scores (except 1.0% NaCl) and raw desirability ratings of 1.0% salt treated steaks were higher than the control. Blends containing NaCl (C1) were darker brown in cooked color than the control; however, no significant differences were found between the control and NaCl formulated steaks for cooked desirability scores. Steaks containing KCl and $MgCl_2$ (C3 and C4) were rated higher for cooked desirability when compared to the control.

Restructured steaks made with KCl, $MgCl_2$ and $CaCl_2$ (except 1% $CaCl_2$ and 1.0% $MgCl_2$) were more desirable and darker red in raw color than blends formulated with NaCl (C5, C6 and C7). These data agree with Schwartz (1975) who reported improved colors in formulations containing nonsodium salts. Hunter A values indicated that increased levels of 1.0% $CaCl_2$, $MgCl_2$ and KCl resulted in a darker red color in raw restructured steaks. Cooked color scores were higher for all $CaCl_2$, $MgCl_2$ and KCl formulations (C5, C6 and C7) as compared to NaCl blends. No significant differences were found between steaks formulated with NaCl or $CaCl_2$ for cooked desirability ratings (C5); however, blends formulated with KCl and $MgCl_2$ were rated more desirable for cooked color than blends formulated with NaCl (C6 and C7).

Data for sensory properties of restructured steaks (Table 2) indicate control restructured steaks were not significantly different from steaks treated with either NaCl or KCl for all sensory traits. However, in data not presented in tabular form the sensory panelists reported that the 1.0% KCl blends had a higher incidence of metallic off-flavors compared to the control blends. Flavor desirability scores and overall satisfaction ratings for C2 and C4 showed blends formulated with 1.0% $CaCl_2$ or $MgCl_2$ were rated lower and less desirable when compared to control formulations. No significant differences were found between the control and all salt treatments for juiciness and tenderness scores. NaCl treated steaks were rated higher than steaks made with $CaCl_2$ or $MgCl_2$ for flavor desirability and overall satisfaction scores (C5 and C7). No significant differences were found between NaCl formulations and blends formulated with KCl for any sensory traits (C6), indicating KCl may be used as a possible substitute for NaCl in restructured beef steaks, although the 1.0% KCl steaks were found to have an increased number of metallic off-flavors that may result in poor consumer acceptability. Data not shown in tabular form indicated no significant differences existed between restructured steaks for bite or cohesiveness scores.

Data not presented in tabular form indicated that the percentage of flavor defects identified in restructured steaks by eight panel members ranged from 16.7% (0.5% $CaCl_2$) to 72.7% (1.0% $MgCl_2$) of all evaluations. An increased incidence of off-flavors occurred as salt increased from 0.5 to 1.0% in all treatments. These results are in agreement with Hand et al. (1982a) and Puolanne and Terrell (1983) who reported that increases in salt levels increased the incidence of off-flavors due to the chloride ion creating a metallic sensation during sensory panel evaluations.

Blends containing 1.0% $CaCl_2$, KCl and $MgCl_2$ had a higher incidence of off-flavors than the control, ranging from 58.3–72.7% of all sensory panel evaluations. These data indicate that the increase in level of added nonsodium salt used in restructured beef steaks resulted in off-flavor defects, especially if added at the 1.0% level. The higher level of nonsodium salts used may contribute to the increased metallic off-flavors. The absence of these off-flavors at lower non-sodium salt levels would indicate that their development may have occurred when 1.0% levels of KCl, $MgCl_2$ and $CaCl_2$ salts were added to the restructured steaks.

No significant differences were found for the textural properties, peak load (shear force), fail energy (work index) or fail elongation (distance required to shear the sample) (Table 3). Peak load values were synonymous with Warner-Bratzler shear force values and were not different between treatments because all lean and fat were flaked prior to steak formulation. The increased binding normally found with increased salt levels did not occur, and the increased added salts failed to extract more myofibrillar proteins. These results could have occurred be-

Table 1—Contrast means for visual traits of restructured steaks formulated with two levels of four chloride salts

	Raw color[a]		Raw desirability[b]		Cooked color[c]		Cooked desirability[d]		Hunter A	
	Salt, %									
Contrasts	0.5	1.0	0.5	1.0	0.5	1.0	0.5	1.0	0.5	1.0
C1 Control vs NaCl	3.6 4.8*	3.6 4.4	4.0 5.0*	4.0 3.0*	5.0 3.6*	5.0 2.6	5.0 5.2	5.0 4.6	9.8 8.7	9.8 10.9
C2 Control vs $CaCl_2$	3.6 5.8*	3.6 4.6*	4.0 5.8*	4.0 5.0*	5.0 4.2	5.0 5.0	5.0 5.4	5.0 5.0	9.8 9.2	9.8 12.0
C3 Control vs KCl	3.6 6.4*	3.6 6.4*	4.0 6.8*	4.0 7.0*	5.0 4.2	5.0 4.4	5.0 6.8*	5.0 6.0*	9.8 10.9*	9.8 12.3*
C4 Control vs $MgCl_2$	3.6 6.0*	3.6 5.6*	4.0 6.5*	4.0 3.6	5.0 4.4	5.0 4.6	5.0 6.0*	5.0 6.0*	9.8 12.3*	9.8 11.2
C5 NaCl vs $CaCl_2$	4.8 5.8*	4.4 4.6	5.0 5.8*	3.0 5.0*	3.6 4.2*	2.6 5.0*	5.2 5.4	4.6 5.0	8.7 9.2	10.9 12.0
C6 NaCl vs KCl	4.8 6.4*	4.4 6.4*	5.0 6.8*	3.0 7.0*	3.6 4.2*	2.6 4.4*	5.2 6.8*	4.6 6.0*	8.7 10.9*	10.9 11.2
C7 NaCl vs $MgCl_2$	4.8 6.0*	4.4 5.6*	5.0 6.5*	3.0 3.6	3.6 4.2*	2.6 5.0*	5.2 6.0*	4.6 6.0*	8.7 12.3*	10.9 12.0

[a] Raw color (3 = moderately brown, 6 = moderately bright cherry red).
[b] Raw desirability (3 = moderately undesirable, 7 = undesirable).
[c] Cooked Color (3 = moderately dark brown, 5 = slightly light brown).
[d] Cooked desirability (4 = slightly undesirable, 6 = moderately desirable).
[e] Hunter A = (red).
* Paired means with the same contrast and salt level are significantly different ($P<0.05$).

Table 2—Contrast means for various sensory panel traits of restructured steaks formulated with two levels of four chloride salts

	Juiciness[a]		Tenderness[b]		Flavor desirability[c]		Overall satisfaction[d]	
	Salt %							
Contrasts	0.5	1.0	0.5	1.0	0.5	1.0	0.5	1.0
C1 Control vs NaCl	6.1 6.0	6.1 6.4	5.9 5.4	5.9 6.3	5.1 5.1	5.1 5.6	5.4 5.3	5.4 5.6
C2 Control vs $CaCl_2$	6.1 5.8	6.1 6.3	5.9 5.5	5.9 5.7	5.1 4.7	5.1 4.0*	5.4 5.3	5.4 4.3*
C3 Control vs KCl	6.1 5.9	6.1 6.4	5.9 5.8	5.9 6.0	5.1 5.0	5.1 5.3	5.4 5.8	5.4 5.5
C4 Control vs $MgCl_2$	6.1 5.5	6.1 5.5	5.9 5.5	5.9 6.0	5.1 5.5	5.1 4.2*	5.4 5.5	5.4 4.5*
C5 NaCl vs $CaCl_2$	6.0 5.8	6.4 6.8	5.4 5.5	6.3 5.7	5.1 4.7*	5.6 4.0*	5.3 5.3	5.6 4.3*
C6 NaCl vs KCl	6.0 5.9	6.4 6.4	5.4 5.8	6.3 6.3	5.1 5.0	5.6 5.3	5.3 5.8	5.6 5.5
C7 NaCl vs $MgCl_2$	6.0 5.5	6.4 5.5	5.4 5.8	6.3 6.3	5.1 5.5	5.6 4.2*	5.3 5.5	5.6 4.5*

[a] Juiciness (5 = slightly juicy, 6 = moderately juicy).
[b] Cohesiveness (5 = slightly cohesive, 6 = moderately cohesive).
[c] Flavor desirability (5 = slightly desirable, 4 = slightly undesirable).
[d] Overall satisfaction (5 = slightly desirable, 4 = slightly undesirable).
* Paired means with the same contrast and salt level are significantly different ($P<0.05$).

Table 3—Contrast means for Instron values of restructured steaks formulated with two levels of four chloride salts

	Instron values					
	Peak load[a]		Fail energy[b]		Fail elongation[c]	
	Salt, %					
Contrasts	0.5	1.0	0.5	1.0	0.5	1.0
C1 Control vs NaCl	4.7 5.1	4.7 5.1	13.4 13.5	13.4 12.9	5.7 5.5	5.7 5.5
C2 Control vs $CaCl_2$	4.7 4.7	4.7 5.0	13.4 14.0	13.4 13.8	5.7 5.7	5.7 5.8
C3 Control vs KCl	4.7 4.4	4.7 4.6	13.4 12.8	13.4 12.6	5.7 5.8	5.7 5.6
C4 Control vs $MgCl_2$	4.7 4.0	4.7 5.1	13.4 13.4	13.4 12.7	5.7 5.8	5.7 5.9
C5 NaCl vs $CaCl_2$	5.1 4.7	5.1 5.0	13.5 13.8	12.9 13.8	5.5 5.7	5.5 5.8
C6 NaCl vs KCl	5.1 4.4	5.1 4.6	13.5 12.8	12.9 12.6	5.5 5.8	5.5 5.8
C7 NaCl vs $MgCl_2$	5.1 4.9	5.1 5.1	13.5 13.4	12.9 12.7	5.5 5.8	5.5 5.8

[a] Expressed as maximum force required to shear a sample, kg.
[b] Expressed as index of amount of work required to shear a sample, cm-kg-force.
[c] Expressed as index of distance required to shear a sample, cm-kg-force.

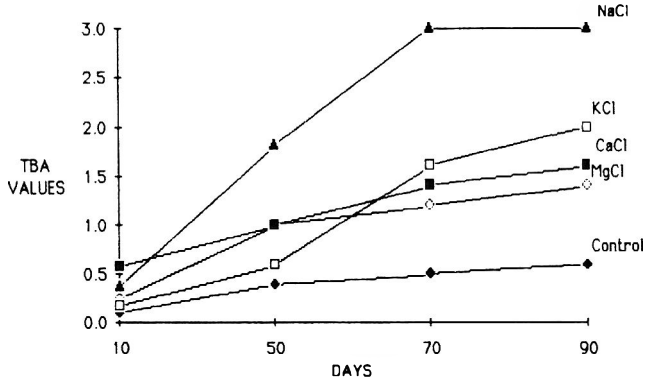

Fig. 1.—Thiobarbituric acid values for the control and salt-treated restructured steaks.

cause the flaking of the meat prior to mixing may have exposed the maximum surface area of the myofibrillar proteins enabling the lower salt levels to extract a similar amount of protein. Thus, the blends form a similar emulsion stability and will have similar textural properties. Additionally, these results indicated that no improvements ($P>0.05$) in binding or cohesion between meat flakes were found when increased levels of salt were added. These data were in agreement with the tenderness ratings by the sensory panel.

Thiobarbituric acid data showed that restructured steaks made with various salts were rancid at the end of 70 days freezer storage time (Fig. 1). These data agree with Huffman et al. (1981) who reported rancidity increased linearly over time with increased salt levels. Control blends were still acceptable for oxidation of lipids at 90 days storage. These results are as expected since the STP will help chelate metal ions and slow the rate of rancidity. NaCl formulated restructured steaks had the highest TBA values compared to all other salt formulations. Although all salt treatments were high, the NaCl treated steaks were at least 1.0 unit more rancid than all other salt formulated steaks. Thus, a need exists to determine the effects of antioxidants on restructured steaks formulated with various salts.

CONCLUSIONS

ADDITIONS of NaCl, KCl, $CaCl_2$ or $MgCl_2$ to restructured steaks resulted in reduced storage times and increased levels of lipid oxidation. Restructured steaks formulated with KCl, $CaCl_2$ or $MgCl_2$ were more desirable in raw desirability scores and these salts enhanced the raw acceptance of substitute salt formulated restructured steaks. $MgCl_2$ and $CaCl_2$ salts added at the 1.0% level caused an undesirable off-flavor in restructured steaks and resulted in lower sensory ratings. Restructured steaks formulated with 0.5% KCl were acceptable for flavor desirability and overall satisfaction scores; however, off-flavor problems still existed in 1.0% salt blends. The need to study the use of KCl in combination with NaCl exists because improved visual and sensory attributes may result when KCl and NaCl salts are used in combination. The improvements in raw visual scores by KCl and the flavor enhancement of NaCl may be important in the final acceptability of restructured steaks by consumers.

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Capillary Gas Chromatography—Mass Spectrometric Analysis of Cooked Ground Beef Aroma

GLESNI MAC LEOD and JENNIFER M. AMES

ABSTRACT

Representative samples of cooked ground beef aroma were isolated onto Tenax TA. Fractionation using capillary GC showed that a cooked meaty odor was associated with only four aroma fractions. These contained relatively high boiling components, and 31 locations on the gas chromatogram were anchored by meaty descriptions using GC odor port assessment. Several of the components identified by GC-MS have not been reported previously from heated beef. They comprise some compounds contributing meaty character e.g. 2-methyl-3-(methylthio)furan and 3-methylcyclopentanone. Several unsaturated alicyclic ketones present resemble the cyclohexenones previously shown to be significant in meaty aroma.

INTRODUCTION

THE AROMA of cooked beef has an intricate composition, and although about 740 of its volatile components have been recorded (Self et al., 1963; Ching, 1979; Wasserman, 1979; Golovnya et al., 1979; Golovnya and Rothe, 1980; Uralets and Golovnya, 1980; Yamaguchi et al., 1980; MacLeod and Seyyedain-Ardebili, 1981; Lee et al., 1981; Hsu et al., 1982; Mottram et al., 1982; Galt and MacLeod, 1983, 1984; Hartman et al., 1983), their relative significance is not clear. The probability is that major components of the aroma are now established. However, most of the analyses reported have involved the use of packed column gas chromatography, and many trace components have eluded identification. On the basis of our current incomplete knowledge, these minor components are the difficult targets for analytical interest in the future. Certainly, there is no known single unique character impact compound for cooked beef aroma, although many workers believe that potent key meaty compounds are present and remain to be identified. Similarly, it is likely that many nonmeaty compounds — which play a rôle by synosmic effects — await characterization. The best opportunity yet available for the more detailed aroma analyses now demanded is currently offered by the relatively high loading capacity and good resolution capability of the recently exploited and robust bonded-phase fused silica capillary columns used for gas chromatography (GC) in conjunction with mass spectrometry (MS).

The present paper reports one such application, the specific objectives of which were: (a) to obtain a valid isolate representative of cooked ground beef aroma, actively avoiding complicated multistage work-up techniques, (b) to fractionate chromatographically, and thereby simplify, the complex aroma, (c) to separate and identify as many as possible of the individual aroma components using capillary GC and capillary GC/MS, (d) to describe sensorially the total aroma isolated, its fractions and also the spectrum of volatiles present, thus assessing the relative sensory contributions of identified components, and in particular, anchoring and identifying if possible any components possessing a meaty odor.

Authors MacLeod and Ames are with the Dept. of Food & Nutritional Sciences, King's College London, Kensington Campus, Campden Hill Road, London W8 7AH, England.

MATERIALS & METHODS

Aroma Isolation

The aroma isolation apparatus is shown in Fig. 1. All glassware was acid-washed and silanised before use. All N_2 supplies (CP grade; BOC Ltd., London, England) were dried and purified using 250g freshly-regenerated molecular sieves 5A and 13X (BDH Chemicals Ltd., Dagenham, Essex, England).

Tenax TA (60-80 mesh, Chrompack UK Ltd., 61 Shrubbery Rd., London, England) was pre-conditioned under a N_2 flow of 50 mL min^{-1} at 340°C for 2 hr. Deactivated glass sampling tubes (20 cm × 4 mm i.d., 6 mm o.d.) were then packed with 200 mg of the Tenax, plugged with silanized glass wool, re-conditioned as just described and capped. Immediately before use, the tubes were further conditioned under N_2 (50 mL min^{-1}) at 300°C for 15 min, cooled to room temperature (20°C), and this latter procedure repeated.

Fresh beef fillet steak (200g) from a local butcher was trimmed of all excess fat and ground to a particle diameter of 4mm. It was cooked with constant stirring in a Teflon-coated electric frying pan at a surface temperature of 104°C for 2 min, followed by 171°C for 6 min. The cooked beef was then transferred into the stoppered sampling flask (g) of Fig. 1. After equilibrating the headspace for 5 min, purified N_2 at 50 mL min^{-1} was passed through the meat maintained in a water bath at 90°C for 24 hr, during which time the cooked beef aroma volatiles were adsorbed on the Tenax TA in each of the four in-parallel sampling tubes (*i*). After sampling, each tube was backflushed with purified N_2 at 50 mL min^{-1} for 2 hr to remove water. A blank experiment (no meat) was performed before each aroma isolation to monitor adequate cleansing of glassware and N_2 purity.

Sensory analysis of total isolate

Using a Pye Unicam (Cambridge, England) Series 104 (Model 64) gas chromatograph, the total aroma desorbed from an isolate obtained as described above was assessed sensorially at an external odor port attached to an *empty* silylated and heated (200°C) short glass GC column (0.3m × 4 mm i.d., 6 mm o.d.). The aroma was described by two assessors experienced in the descriptive sensory analysis of odors. The Tenax tube (aligned in the opposite direction to that used for sampling) and a three-way valve were incorporated into the carrier gas line at the column inlet by means of Swagelok unions. Using the valve, air was flushed out of the tube by N_2 gas (30 mL min^{-1}) for 5 sec to prevent Tenax decomposition on subsequent heating. The Tenax was then heated to 250°C over 1 min and held at 250°C for an additional minute, while venting the N_2 to atmosphere. The N_2 was then re-diverted through the Tenax, flushing the aroma volatiles into the GC column. The aroma was split in a 100:1 ratio at the column exit with the major portion passing through a silylated and heated (200°C) line to the odor port.

Capillary gas chromatography

The isolated aroma was analyzed by capillary GC using a Perkin Elmer (Beaconsfield, Bucks, England) Sigma 2B instrument and a fused silica bonded-phase DB5 capillary column (60m × 0.32 mm i.d. × 1 μm film; J & W Scientific Inc. from Jones Chromatography Ltd., Colliery Rd., Llanbradach, Mid. Glam., Wales). The normal capillary injector system was not used. Instead, the packed column injector was operated at 30°C, and the Tenax tube was attached to the

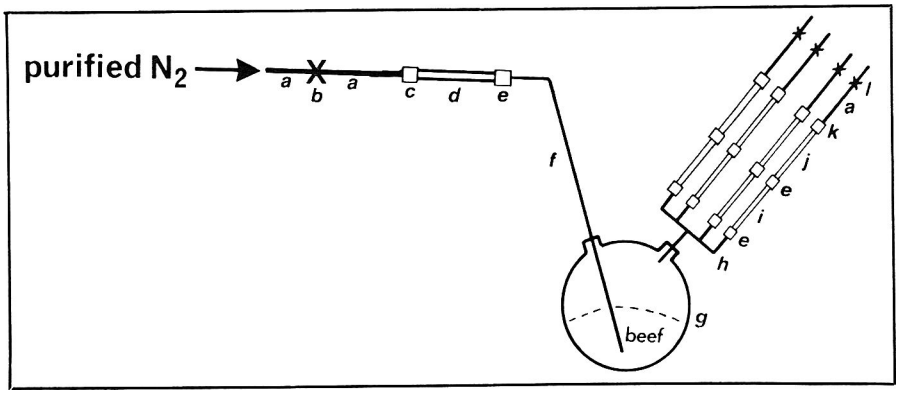

Fig. 1—The Aroma Isolation Apparatus a = teflon tubing (⅛" o.d.); *b* = needle-valve; *c* = stainless steel union (⅛" - ¼"); *d* = conditioned Tenax TA (200mg) purifier tube (6mm o.d. glass); *e* = teflon barrel union (¼" - ¼"); *f* = glass tube (6mm o.d., 4 mm i.d.); *g* = sample flask (500mL) in water bath at 90°C; *h* = glass manifold (6 mm o.d., 4mm i.d.); *i* = glass sampling tubes (20cm × 6mm o.d., 4 mm i.d.) of Tenax TA (200mg) pre-conditioned; *j* = silica gel drying tubes (6mm o.d., 4mm i.d.); *k* = union (¼" - ⅛"); *l* = flowmeters with integral needlevalves.

He carrier gas line and flushed with He at 250 mL min^{-1} (1 psi) for 5 sec to remove air. The remote end of the tube was then immediately attached to the GC column, the first 0.3m of which formed a U-trap cooled in liquid N_2. The He pressure was increased to 20 psi (~ 3 mL min^{-1}), and the Tenax heated to 250°C over 1 min and held at 250°C for an additional minute. During this time, the He transferred the heat-desorbed volatiles into the cold trap, and the He flow was maintained for 10 min after heating. The liquid N_2 was then removed, the cold trap was fed into the GC oven, and the temperature program started, i.e., 30°C (initial) increased at 1°C min^{-1} to 55°C and then at 2°C min^{-1} to a final temperature of 200°C and held until all peaks had emerged. Other relevant GC conditions were: flame ionization detector at 250°C; N_2 (MUG) 30 mL min^{-1}; H_2 30 mL min^{-1}; air 500 mL min^{-1}; attenuation 1 × 64, i.e., 32 × 10^{-11}. A full scale deflection. Retention times and peak areas were recorded by a Hewlett-Packard (Slough, Bucks, England) integrator (Model 3370B). Peak areas were expressed as a percentage of the total peak area, i.e., relative percentage abundance (RPA).

Sensory analysis of resolved isolate

The isolate obtained and chromatographed as described above was assessed sensorially at the DB5 column outlet during chromatographic separation using the technique of GC odor port assessment (GC-OPA). The column effluent was split in a 3:1 ratio by means of an outlet splitter (SGE Ltd., Milton Keynes, England) containing a four-holed Vespel (Chrompack UK Ltd.) ferrule, such that four equal lengths of deactivated fused silica (0.4m × 0.32 mm i.d.) led from the ferrule, one length to the GC detector and the other three parallel lengths together via an unused heated (250°C) detector line to an external odor port. The odors of the separated components were described by two assessors experienced in the descriptive sensory analysis of odors.

Fractionation of the isolate

Volatile components from the isolate were heat-desorbed as described under "Capillary Gas Chromatography" onto four in-parallel fused silica DB5 capillary columns (60m × 0.32 mm i.d. × 1 μm film) in the GC oven. During chromatographic separation and using the conditions described already, six fractions were trapped on a sequential basis from three column exits. A zero dead volume union contained a four-holed Vespel ferrule at the column inlets and a three-holed Vespel ferrule at the column outlets. The fourth column was directly attached to the GC detector to monitor the cut-off points defined in Tables 1–3. Each fraction was collected on a single clean conditioned Tenax TA (200 mg) tube (pre-equilibrated in solid CO_2 for 10 min) via an unused heated (250°C) GC detector port. Each fraction was then purified by repeating the above procedure once more. Fractions were analyzed sensorially and chemically.

Capillary gas chromatography-mass spectrometry

Components were identified as far as possible by gas chromatography-mass spectrometry (GC-MS) analysis of isolates and of fractions using a Perkin Elmer Sigma 3 GC interfaced via a single-stage all glass jet separator at 250°C to a Kratos MS25 instrument (Kratos Analytical, Manchester, England) linked on-line to a Kratos DS 50S data processing system and equipped with a computer-controlled multipeak monitoring (MPM) unit. The same GC conditions as described above were employed. Significant operating parameters of the mass spectrometer were: electron ionization potential, 70 eV; ionization current, 100 μA; source temperature, 200°C; accelerating voltage, 4 kV; resolution, 600; scan speed, 1 sec $decade^{-1}$, repetitive throughout the run.

RESULTS & DISCUSSION

PRELIMINARY EXPERIMENTS comparing the artefact background on thermal desorption of pre-conditioned Tenax GC vs. Tenax TA (both 60-80 mesh, Chrompack UK Ltd., London, England) showed that superior blank chromatograms were obtained from Tenax TA (MacLeod and Ames, 1986a). After evaluating a range of conditioning parameters, the most efficient procedure which minimized background on heat desorption was as described in the Experimental Section. Experience showed that the additional brief conditioning treatment immediately before use was particularly beneficial and the excellent baseline chromatogram obtained from Tenax TA (200 mg) — conditioned and chromatographed as described above — has been reported previously (MacLeod and Ames, 1986a).

From a sensory viewpoint, the cooked beef after sampling was described as "very well-cooked with a strong meaty-roast aroma character." The total aroma desorbed from the isolate and assessed sensorially was described by the odor qualities: sweet, strongly meaty, meaty-roast, burnt, musty and buttery. It follows, therefore, that the aroma isolated was representative of the cooked ground beef analyzed and, in particular, that cooked meaty character was being sampled onto the Tenax and desorbed from it under the analytical conditions used. By the same sensory analysis technique, it was shown that any background aroma of nonmeat origin, i.e., from a heat-desorbed conditioned blank Tenax tube, was negligible (a slight cardboard-like aroma could just be discerned) and also that backflushing the aroma isolate under the conditions described caused no significant sensory changes.

The results obtained from several capillary GC and GC-MS analyses of isolates and of fractions are presented in Tables 1-3. A blank isolation (no meat) showed that the components entered in Table 3 were artefacts. They did not originate from the Tenax TA *per se* under the conditions used (MacLeod and Ames, 1986a) but were concentrated on the adsorbent from the purified CP N_2 sweep-gas during sampling and could not be minimised further. They are mainly aliphatic, alicyclic and aromatic hydrocarbons and chlorinated compounds.

Fractionation achieved simplification of the complex isolate, and the technique employed was based on the "bunch chromatography" system described by de Nijs et al. (1981). In this case it was possible to avoid overloading each GC column since the sample input was split four-ways. This meant improved efficiency of chromatography and fractionation such that only one re-fractionation was required for purification of each fraction, - the validity and purity of which were checked chromatographically before further analyses.

Table 4 summarizes the sensory properties of each fraction Fa → Ff, showing clearly that Fa and Fb did not possess meaty

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Table 1—Volatile compounds identified in isolates and fractions

Component[a]	t_R (min)	Kováts index (lit.)[b]	MS ref[c]	RPA[d]	Odor quality (GC-OPA)	Fraction[e]
Carbon dioxide	2.85		A	tr		
water	2.90		A	tr		
hydrogen sulfide	3.08		A	tr		
carbonyl sulfide	3.08		A	tr		
*methylpropane	3.40		A	tr		
acetaldehyde	3.57	363	A	0.02		
butane	3.70	400	A	tr		
methanethiol	3.95		A	0.05	cooked cabbage	
trimethylamine	4.13		A	0.05		
ethanol	4.48	500	A	tr		
methylbutane	5.10		A	0.02		
acetone	5.18	530	A	tr		
sulfur dioxide	5.25		A	tr		
*diethyl ether	5.28	572	AB	tr		
*ethyl vinyl ether	5.30		A	tr		
*methylpropene	5.35		A	tr		
*penta-1,3-diene	5.40		A	tr		
carbon disulfide	5.70		A	0.02		
propan-1-ol	5.95	535	AB	tr		
methylpropanal	6.32	500	A	tr		
2-methylpentane	6.72		A	tr		Fa
3-methylpentane	6.75		A	tr		
but-2-enal, i.e., crotonal	7.00		A	tr		
butanedione, i.e., diacetyl	9.40	575	AB	0.05	buttery, caramel	
butanone	9.70	579	A	0.01		
hexane	9.82	600	A	tr		
2-methylfuran	10.07	614	AB	tr		
ethyl acetate	10.15	595	AB	tr		
*methyl propanoate	10.90	611	A	tr		
*methylcyclopentane	11.78		A	tr		
3-methylbutanal	14.60	649	A	9.50	green, fragrant, chemical solvent	
benzene	14.97		A	tr		
cyclohexane	15.10	677	A	tr		
2-methylbutanal	15.60		A	9.50		
acetic acid	15.95		A	0.10		
thiophen	16.15	650	ABC	0.02		
*hydroxypropanone	17.40		A	tr		
*pent-1-en-3-one	17.73		A	tr		
pentan-2-one	17.90	672	AB	0.05		
heptane	19.00	700	AB	2.50		
pentane-2,3-dione	19.00	681	AB	tr	buttery, caramel	
pentanal	19.50	694	A	0.01		
3-hydroxybutanone, i.e., acetoin	21.20	697	AB	1.75		
2-ethoxyethanol	21.75		A	tr		
pyrazine	22.50	739	AB	tr		
thiazole	22.70	715	AC	tr		
pent-3-en-2-one	23.53		A	0.05		
4-methylpentan-2-one	23.62	725	AB	0.05		
*2-methylbut-2-enal	24.30		A	0.10		
methyl propyl sulfide	24.40		A	tr		
dimethyl disulfide	24.60	730	A	0.05		
pyridine	25.12	695	AC	0.25		
3-methylpentan-2-one	26.02		A	0.01		
*4-methyl-2,3-dihydrofuran	26.83		A	0.02		
pyrrole	27.27		AC	0.10		
2-methylheptane	27.65		A	0.01		Fb
methylbenzene, i.e., toluene	28.20		A	0.05		
propanoic acid	28.50		A	0.05		
pentan-1-ol	29.20	756	AB	1.25		
2-methylthiophen	29.70	775	A	0.05		
*pentane-2,4-dione	30.32		A	0.05		
*cyclopentanone	30.83	805	A	tr		
hexan-2-one	31.43	772	AB	0.20		
octane	32.70	800	AB	4.25		
hexanal	32.93	780	AB	6.50	green, grassy	
2-methyltetrahydrofuran-3-one	34.20		AC	12.00	caramel, green	
2-propylfuran	34.85	782	AB	tr		
4(or 5)-methylthiazole	34.98	800 (4-Me)	ABC	0.10		
*2-methylpent-2-enal	35.40			tr		
methylpyrazine	35.85	805	ABC	4.00	green, sl. fragrant	
2-furancarboxaldehyde, i.e., furfural	36.35	815	ABC	0.02		
*hexan-3-one	36.65	767	AB	0.02	sl. caramel	
*2-methylcyclopentanone	37.00	810[f]	A	0.05		
chlorobenzene	37.23		A	tr		
*3-methylcyclopentanone	37.50	820[f]	A	0.02	*sl. meaty*, fatty, fruity, caramel	

Table 1—Continued

Component[a]	t_R (min)	Kovàts index (lit.)[b]	MS ref[c]	RPA[d]	Odor quality (GC-OPA)	Fraction[e]
*5(or 4)-methylthiazole	37.93		A	0.05	very sickly	
2-methylpyrrole	38.25		A	0.02		
2,4,5-trimethyloxazole	38.50	829	BC	0.10		
butanoic acid	38.67		A	0.01	cheese-like, sweaty, rancid	
*5-methylhexan-2-one	39.37	825	AB	0.05		
ethylbenzene	39.75		A	0.20		
2-furanmethanol	40.95		AC	tr	*meaty roast*	
hexan-1-ol	41.80	858	AB	0.75	fragrant	
pentanoic acid	42.25		A	0.02	*meaty*	
*2-methyl-2-thiazoline	42.50		A	0.05	buttery, caramel	
bis(methylthio)methane	43.40		D	tr		
vinylbenzene, i.e., styrene	43.60		A	tr	caramel, roasted, *sl. meaty*	Fc
heptan-2-one	43.75	872	AB	1.00		
cyclohexanone	44.00	875	AB	0.02	green, fragrant, stale	
nonane	44.60	900	AB	0.10	sweaty, dull *meat fat*	
heptanal	45.05	883	AB	2.75	caramel, buttery, fragrant, *cold meat fat*	
*2-methylcyclopent-2-enone	45.36		A	0.05	sweet, sickly, green	
2-ethylpyridine	45.55		A	0.10		
2,5(and/or)2,6-dimethylpyrazine	46.20	893(2,5-) 895(2,6-)	ABC	8.00	musty, *meaty roast*, green	
ethylpyrazine	46.60		AC	0.50	chicory	
4-hydroxybutanoic acid lactone, i.e., γ-butyrolactone *or* butanolide	46.70	885	A	tr		
2,3-dimethylpyrazine	47.00	900	ABC	0.50	*meaty*, dull	
dimethyl sulfone	47.60		A	0.01		
*methyl hexanoate	47.60	906	AB	tr		
vinylpyrazine	48.05		A	0.05	stale garlic	
*butylcyclopentane	48.78		A	0.01	toasted	
*2-methyl-3-(methylthio)furan	50.20	959[f]	EF	0.10	*meaty, not roasted*, pleasant	
propylbenzene	50.83		A	0.02	nutty, Chinese lettuce	
benzaldehyde	51.60	947	AB	3.25	nutty, almonds	
*3-ethylpyridine	51.80		A	tr		
dimethyltrisulfide	52.20	952	A	0.05	rotting green vegetables, cabbage	
heptan-1-ol	52.65	957	AB	0.75	sweet, fruity, butterscotch	
*1,3,6-octatriene	53.08		A	0.05	fragrant, heated butter	
oct-1-en-3-ol	53.70	968	AB	1.00	green, sickly	
phenol	54.00	1002	AB	tr	boiled new potatoes	
octan-2-one	54.40	991	AB	0.75	caramel	
2-pentyifuran	54.55	983	AB	0.01		
decane	55.03	1000	AB	tr		Fd
2-ethyl-5(and/or)6-methylpyrazine	55.26		A	3.75	sickly, fruity, toffee	
2-ethyl-3-methylpyrazine	55.70	987	AB	5.00	green, fragrant, floral, fresh nuts	
octanal	56.00	985	AB	0.05	sweet, fruity, nutty	
trimethylpyrazine	56.20	985	ABC	0.01	sweet, caramel	
2-methyl-5-(and/or)6-vinylpyrazine	57.03		AC	0.50		
2-acetylthiazole	57.35	995	BC	0.20	caramel	
limonene	57.85	1030	AB	0.01		
2-acetylpyridine	58.10	1014	ABC	tr	sweet, fruity	
indene	58.78		A	0.05		
hepta-2,4-dienal	60.80		A	0.10		
2-acetylpyrrole	60.95	1050	AC	tr	oily, fatty, putty	
acetophenone	61.00	1048	AB	0.01		
octan-1-ol	61.10	1061	AB	0.10		
2-acetyl-l-methylpyrrole	61.37	1055	AC	0.01	*sl. meaty* boiling vegetables	
2,5-dimethyl-3-ethylpyrazine	61.80		AC	0.02	nutty, walnuts	
tetramethylpyrazine	62.90	1069	ABC	2.00	walnuts, green	
nonan-2-one	63.17	1093	AB	tr	boiled milk, sl. burnt	
undecane	63.60	1100	AB	0.50		
nonanal	63.80	1087	AB	0.50	fruity, fragrant, apricot, peach	
2-methyl-5(and/or)6-(prop-l-enyl) pyrazine	63.95		A	0.05	burnt, fatty	
2-acetyl-2-thiazoline	64.62		C	0.05	floral	
6,7-dihydro-(5H)cyclopentapyrazine	65.00		C	0.02	green, fatty, dull	
5-methyl-6,7-dihydro-(5H) cyclopentapyrazine	68.30		C	0.02		
*camphor	69.05	1136	AB	0.02	sweet, fragrant	
2,6-dimethylstyrene	69.33		A	0.01	fresh, green	
2,3-diethyl-5-methylpyrazine	69.55		A	0.10	crushed leaves, bitter	
2,6-diethyl-3-methylpyrazine	69.80		A	0.25		
2-ethyl-3,5,6-trimethylpyrazine	70.03		A	0.25		
2,5-diethyl-3-methylpyrazine	70.70		G	0.05	sweet, caramel, cakes	
*4-methylene-3,5,5-trimethylcyclohex-2-enone	71.15		H	0.05	cats' urine, unpleasant	
methyl phenylacetate	71.90	1154	AB	tr		Fe

Table 1—Continued

Component[a]	t_R (min)	Kovats index (lit.)[b]	MS ref[c]	RPA[d]	Odor quality (GC-OPA)	Fraction[e]
dodec-1-ene	72.20		A	0.05		
decan-2-one	72.50	1176	AB	0.75	caramel, fragrant	
naphthalene	72.70		A	0.50	fragrant	
dodecane	72.85	1200	AB	0.10	fragrant, floral, geranium	
decanal	73.10	1188	AB	tr		
4-hydroxyoctanoic acid lactone, i.e., γ-octalactone	78.30	1225	AB	0.10		
undecan-2-one	80.90	1276	AB	tr	sweet, fragrant, chemical solvent	
tridecane	81.20	1300	AB	0.50	*burnt meat*	
*2-butyl-3,5-dimethylpyrazine	83.15		A	0.10	fragrant	
					meat drippings	
					musty, moldy	
					- - - - - - - -	Ff
						to end

[a] Asterisked components have not been reported previously from heated beef.

[b] Kovats index lit. for nearest stationary phase (OV101), confirming the general elution sequence; Jennings and Shibamoto, 1980.

[c] MS reference: A–Anonymous (1983); B–Jennings and Shibamoto (1980); C–Vernin (1982); D–Cuer et al. (1979); E–Tressl and Silwar (1981); F–MacLeod and Ames (1986b); G–Friedel et al. (1971); and H–Demole (1984).

[d] Relative percentage abundance (RPA) values have been corrected as follows: 5% quoted to nearest 0.5%; 0.25–5% quoted to nearest 0.25%; < 0.25% quoted as 0.25, 0.2, 0.1, 0.05, 0.02, 0.01%. "tr" indicates that the component was present in trace amount but no accurate peak area measurement was possible.

[e] The identified chemical composition of each of the six fractions (Fa → Ff) collected is given by the combined identities indicated in Tables 1–3. Fraction cut-off points were as follows: Fa after thiophen at t_R 16.15; Fb after hexanal at t_R 32.93; Fc after a terpenoid at t_R 48.55 (see Table 2); Fd after 2-methyl-5 (and/or 6)-(prop-1-enyl)pyrazine at t_R 63.95; Fe after 2-butyl-3,5-dimethylpyrazine at t_R 83.15; Ff at 135 min.

[f] Kovats index determined on DB5 stationary phase; see also MacLeod and Ames (1986b).

odors. Therefore, the components identified in Fa and Fb (see Tables 1-3) are relatively unimportant in that — in combination — they impart no meaty character, although they act as "modifiers" and intermediates. Furthermore, as shown by GC-OPA analysis, none of the GC peaks of Fa and Fb possessed a meaty odor (see Tables 1-3), suggesting also that meaty character impact compounds are lacking in these two fractions which elute early in the total gas chromatogram.

Each of the remaining fractions, i.e., Fc, Fd, Fe and Ff did possess a cooked meaty odor (see Table 4), and the most convincing result of the GC-OPA analysis was that definite meaty notes were associated with 31 specific areas of the gas chromatogram. Therefore, meaty character impact compounds would appear to be present, and the anchored meaty locations are underlined in Tables 1-3. They predominate in the last two-thirds of the gas chromatogram, and are therefore relatively high boiling components. It is not surprising that many of the GC peaks are minute or non-existent and remain unidentified. Identities do exist for several, but it is possible that the true meaty note in these cases is due to a trace coeluent.

Of the valid cooked beef aroma components identified, the asterisked compounds of Tables 1 and 2 have not been reported previously from heated beef (Self et al., 1963; Ching, 1979; Wasserman, 1979; Golovnya et al., 1979; Golovnya and Rothe, 1980; Uralets and Golovnya, 1980; Yamaguchi et al., 1980; MacLeod and Seyyedain-Ardebili, 1981; Lee et al., 1981; Hsu et al., 1982; Mottram et al., 1982; Galt and MacLeod, 1983, 1984; Hartman et al., 1983). They comprise some interesting compounds contributing meaty character, as discussed below.

Some of the alicyclic ketones identified are unsaturated derivatives which resemble the cyclohexenones identified by Flament et al. (1978) on heating a meat aroma precursor preparation obtained from raw meat. In particular, the identified 4-methylene-3,5,5-trimethylcyclohex-2-enone is the methylene derivative of isophorone reported in the meat aroma described by Flament et al. (1978) and also in the volatiles of a yeast extract composition by Ames and MacLeod (1985). Flament et al. (1978) state that "these unsaturated alicyclic ketones have interesting sensory properties; when combined with an alkylpyrazine, they cause flavor enhancement, improvement or modification; in particular they impart a roasted, grilled, sometimes earthy note or a more pronounced taste of meat . . . . . ". The use of cyclopent-2-enones (King and Smith, 1976) and cyclohex-2-enones (Flament, 1974) in meat flavors has been patented.

The association in Table 1 of 3-methylcyclopentanone (not previously reported from heated beef) with meatiness agrees with a recent report by Nishimura et al. (1980). They obtained a reaction product mixture which they described as "somewhat meaty" by heating 2-hydroxy-3-methylcyclopent-2-enone, i.e., cyclotene, with hydrogen sulfide. Two of the volatile reaction products were identified as 2-methylcyclopentanone and 3-methylcyclopentanone, both of which were given the odor description "roasted beef." Both compounds were also present, and in larger amounts, from a model system of heated cyclotene/H_2S/NH_3. Their formation in meat by similar reactions is likely, since cyclotene is a thermal degradation product of sugars and a Maillard reaction product (Hodge, 1967; Tressl et al., 1979), although it has not been reported from heated beef.

Another meaty component identified in this work and worthy of note is 2-methyl-3-(methylthio)furan (MacLeod and Ames, 1986b). The mass spectrum obtained agreed well with that reported by Tressl and Silwar (1981). However, to confirm its identity, the compound was synthesized using the method of Cederlund et al. (1977), and the mass spectrum of the synthesized compound was an excellent match with that obtained from the cooked beef (MacLeod and Ames, 1986b). In cooked beef aroma, this compound could arise from the reaction of methanethiol with 2-methylfuran-3-thiol which, itself, has a meaty odor and is a thermal degradation product of thiamin (van der Linde et al., 1979). It is also possible that this thiol is generated during the reaction of 4-hydroxy-5-methyl-3(2H)furanone with H_2S, based on the postulated pathways published for this reaction (van den Ouweland and Peer, 1975). Meat flavor patents incorporating our identified 2-methyl-3-(methylthio)furan do exist, e.g., (IFF Inc., 1979), and both the 2,3- and the 2,5- isomers have been reported in the volatiles of roasted coffee (Tressl and Silwar, 1981). The odor thresholds in water of 2-methyl-3- (methylthio) furan and of 2-methyl-3-(methyldithio)furan are 0.05 and 0.01 ppb respectively, and at concentrations < 1 ppb, both possess meaty aroma; at higher concentrations they deliver a thiamin-like note (Tressl and Sil-

Table 2—Volatile compounds partially characterized and tentatively identified in isolates and fractions

Component[a]	t_R (min)	Kováts index (lit.)[b]	MS ref[c]	RPA[d]	Odor quality (GC-OPA)	Fraction[e]
a C_6 alkadiene	13.10		A	tr		Fa
a hexenal	22.37		AB	tr		Fb
a C_7 ketone (M114)	33.35		A	tr		
an octene (M112)	33.56		A	0.10	fatty, *cold meat fat*	
?an octene	34.70		A	tr	caramel, green	
a methylpyridine	35.28		AC	0.01		
*a methylpyrrole	39.15		A	tr		
*a methylpyridine	40.35		AC	0.10	caramel	Fc
a xylene	40.43	860 (*p*)	A	tr		
? 2-ethylthiophen	40.55	861	AB	0.05	*meaty-boiled* cats' urine	
*a methylpyridine	41.10		AC	tr		
a xylene	41.25	863 (*m*)	A	0.10	*meaty*	
*? an octatriene	43.20		A	0.01		
a dimethylpyridine	43.68		AC	0.02	caramel, roasted, *sl. meaty*	
a C_3 alkyl benzene	47.25		A	0.01	sweet, sickly, musty, *meaty*	
a br. ch. C_7 alcohol	47.45		A	0.01		
*propyl (or *iso*propyl) cyclohexane	47.83		A	0.02		
a terpenoid (M136)	48.55		A	0.10	caramel	
*a dimethylpyridine	48.95		AC	0.05	*meaty*	
*a dimethylpyridine	49.28		AC	tr	green, oily, fatty	
*? 4,5-dimethyl-2-ethyloxazole	49.50	914	B	0.02	*meaty*, sl. roasted, pleasant caramel	
a C_8 alkan-2-one	51.03		A	0.20	green, nutty *meaty, sl. roasted*	
a C_3 alkyl benzene	51.95		A	0.05		
3(or 4)-vinylpyridine	52.32		A	0.01		
a C_3 alkyl benzene	53.08		A	0.02	fragrant, heated butter	
an alicyclic hydrocarbon	53.40		A	0.01		
a br. ch. hydrocarbon	53.90		A	0.05	boiled new potatoes	
a trimethylbenzene	54.80		A	0.20		
? a pyrrole (M123)	55.40		ADE	tr		
a terpenoid (M136)	56.28		A	0.02	sweet, caramel	
a dichlorobenzene	56.50		A	0.05		
? a cyclohexane deriv.	56.80		A	0.01	*meaty*	
a C_3 alkyl benzene	57.45		A	tr	caramel	Fd
*? 2(or 3)-(methylthio)thiophen	57.75		A	0.02		
a methylstyrene *or* indan	58.30		A	1.75	sweet, fruity	
a cyclohexane deriv.	58.40		A	tr		
a cyclohexane deriv.	58.78		A	tr		
a methylpropylbenzene	59.10		A	0.01	caramel unpleasant, green, fragrant, sap-like	
a C_4 alkyl benzene	60.00		A	0.01		
?a cyclohexane deriv.	60.00		A	0.02		
*a br. ch. C_{11} hydrocarbon	60.40		A	0.05	flat, dull herbal	
a dimethylethyl *or* a diethylpyrazine	62.12		AC	2.50	fruity, fragrant	
a dimethylethyl *or* a diethylpyrazine	62.60		AC	0.01	*meaty* cats' urine	
*? l-*iso*butyl-2-methylpyrrole	62.95		C	tr	walnuts, green	
*an ethylmethylpyridine	63.35		AC	0.05	oily, fatty	
a C_4 alkyl pyrazine	63.35		A	0.02	burnt, fatty	
*a trimethylcyclopent-2-enone	64.30		A	0.10	burnt, charred, iron-scorch	
*an ethyl (or dimethyl) vinylpyrazine *or* a methylpropenylpyrazine	65.30		A	tr	green, fatty, dull	
a tetramethylbenzene	65.55		A	tr		
*? an acetylpyrrole	65.97		AC	0.20	fragrant	
?a br. ch. C_{12} hydrocarbon	66.60		A	0.05	*meaty roast*	
?a C_4 alkyl benzene	66.60		A	0.05		
an alcohol	66.95		A	0.05		
*? a C_5 sub. pyrrolidine (M141)	67.80		A	0.02	sweet, roasted, caramel	
*4 (or 5)-methylindan	68.05		A	0.05	*sl. meaty*, oily, fatty	
*a methylindene	69.33		A	tr	fresh, green	
*? a methylpropenylpyrazine	70.15		A	0.01	dock leaves very fragrant	
a benzenoid	71.28		A	0.05	*meaty-boiled*	
a sub. cyclohex-2-enone	71.65		F	0.01	herbal, green	
*a nona-2,4-dienol	71.90		A	0.20		
a terpenoid	72.05		A	tr		
*a dimethylindan	72.20		A	0.02		Fe
*a dimethyl*iso*propylbenzene	73.60		A	0.25	sweet, floral, fragrant	
?a dimethyl-6,7-dihydro-(5H)-cyclopentapyrazine	73.93		C	0.20		
a C_4 alkyl dimethylpyrazine	74.15		A	tr		
*a dimethylindan	74.20		A	tr		
?an acetylethylpyrazine	74.80		G	0.01	linseed oil, paint sickly, fragrant	

Table 2—Continued

Component[a]	t_R (min)	Kováts index (lit.)[b]	MS ref[c]	RPA[d]	Odor quality (GC-OPA)	Fraction[e]
?an acetylethylpyrazine	75.50		G	0.02	unpleasant	
*? 3,5-dimethyl-4-dimethylaminophenol	76.12		A	0.02	fragrant, sl. green, fatty stale mint sauce oily, fatty fruity *burnt drippings* green, bitter, dock sl. fragrant	
*a dimethylindan	78.45		A	tr		
a br. ch. C_{13} hydrocarbon	78.75		A	0.05	sweet, fruity, floral, fragrant	
? 2-hexylthiophen	79.60	1256	AB	0.02		
? a C_6 alkyl pyrazine	81.70		A	0.02	burnt	
? a hydrocarbon	82.00		A	0.05	*meaty*	
?a C_6 alkyl pyrazine	85.70		A	tr	fragrant	Ff to end

[a,b] See Table 1 footnotes.
[c] MS references: A–Anonymous. (1983); B–Jennings and Shibamoto (1980); C–Vernin (1982); D–Tressl et al. (1977); E–Shigematsu et al. (1972); F–Demole (1984); G–Kinlin et al. (1972).
[d,e] See Table 1 footnotes.

Table 3—Volatile compounds established as artefacts

Component[a]	t_R (min)	Kováts index (lit.)[b]	MS ref[c]	RPA[d]	Odor quality (GC - OPA)	Fraction[e]
dichloromethane	5.65		A	tr		Fa
trimethylsilanol	6.77		A	tr		
trichloromethane	11.05		A	tr		
1,1,1,-trichloroethane	14.15		A	tr		
an alicyclic hydrocarbon	50.37		A	0.05		Fd
a cyclohexane deriv.	56.20		A	0.02	sweet, caramel	
butylcyclohexane	57.85		A	tr	caramel	
a cyclohexane deriv.	58.47		A	0.02	sweet, caramel	
a diethylbenzene	58.90	1070 (*m*)	A	tr		
an alicyclic hydrocarbon	58.90		A	tr		
5-methylspiro[3,5]-nonanone	67.15		A	0.02		Fe
trans-decal-2-one	67.40		A	tr	burnt, charred, dock leaves, dandelions	
pentylcyclohexane	67.48		A	tr		
a C_5 alkyl benzene	67.60		A	tr	sweet, roasted, caramel	
a diethylmethylcyclohexane	67.65		A	tr		
1,2,3,4-tetrahydronaphthalene	70.90		A	tr	sweet, fragrant	
2-methyl-1,2,3,4-tetrahydronaphthalene	75.15		A	0.01	*meaty roast, sl. meat extract*	
4-methylcinnoline	75.50		A	tr	unpleasant	
hexylbenzene	79.00		A	0.05	sickly, unpleasant	
a methyl-1,2,3,4-tetrahydronaphthalene	79.95		A	0.01	fragrant	
1(or 2)-methylnaphthalene	82.60		A	tr	*meaty*	
2(or 1)-methylnaphthalene	86.00		A	0.01		Ff to end
?a benzylquinoline	86.60		A	tr		
an alicyclic hydrocarbon	86.90		A	tr		
a hydrocarbon	88.20		A	0.10	*meaty, meat extract*	
a br. ch. C_{14} hydrocarbon	88.70		A	tr	*gravy* *meat extract*	

[a] All components listed in this Table were present in a blank isolate (no meat) and are therefore artefacts. It is possible that the odors perceived on GC-OPA of isolates are due to trace coeluents.
[b,c,d,e] See Table 1 footnotes.

war, 1981). Both compounds were recently identified in the volatiles of a yeast extract composition (Ames and MacLeod, 1985), - the empirical use of which for simulated meat flavorings is established. Several furan derivatives which are closely related to our identified 2-methyl-3-(methylthio)furan also possess potent meaty aromas (Evers et al., 1976; Tressl and Silwar, 1981; MacLeod, 1984; MacLeod and Ames, 1986b) and, for example, the odor threshold of *bis*-(2-methyl-3-furyl)disulfide has been recorded recently as 2 parts per 10^{14} parts water (Buttery et al., 1984). This is one of the lowest odor thresholds presently known and is about one hundred times lower than that of 2-*iso*butyl-3-methoxypyrazine, the well-known potent character impact compound of bell peppers (Buttery et al., 1984). It follows therefore that only minute traces of these types of compounds need be present for them to be aroma effective, creating enormous analytical difficulties for their detection.

Table 4—Sensory properties of collected fractions[a]

Fa	NOT meaty, sweet, sickly, buttery, sl. burnt. musty.
Fb	NOT meaty, oily, fatty, sl. musty.
Fc	*meaty-roast*, fatty, burnt, toasted, buttery, caramel.
Fd	*meaty*, fruity, fragrant, nutty, musty heated milk.
Fe	*meaty-roast*, burnt, nutty, musty.
Ff	*meaty-roast*, roasted cereal, sweet.

[a] See Table 1, footnote e.

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Effects of Various Phosphates on the Palatability, Appearance and Storage Traits of Flaked and Formed Restructured Beef Steaks

M. F. MILLER, G. W. DAVIS, S. C. SEIDEMAN, C. B. RAMSEY, and T. L. ROLAN

ABSTRACT

Seven blends (9.1 kg raw meat) were formulated with 0.75% NaCl and 0.25% or 0.5% sodium tripolyphosphate (STP), sodium hexametaphosphate (SHMP) or sodium acid pyrophosphate (SAPP). Steaks from all blends were "slightly" to "moderately" desirable in flavor and overall satisfaction scores. Steaks made with STP had higher shear force values and work required to shear values than all other restructured steaks indicating an increase in binding occurred with added STP. Restructured steaks formulated with 0.5% STP or SHMP had more desirable raw color scores than the control or steaks made with SAPP. Storage life of all phosphate added restructured steaks was past 90 days frozen storage, and steaks containing STP had thiobarbituric acid values below 1.0 at 120 days storage.

INTRODUCTION

THE BINDING of meat pieces into a single meat mass by flaking, mixing and pressing has been utilized for several years (Moore et al., 1976). The agitation of flaked meat particles in the presence of salt and phosphates enhances the extraction of the salt-soluble proteins to bind adjacent meat surfaces. Salt and phosphates may be added to preblends and to meat batters during chopping (Puolanne and Terrell, 1983). Phosphates enhance water binding capacity (Hamm, 1970), and their use in restructured steaks may increase the water binding capacity in blends with low salt content.

Phosphates added to processed meat products improve texture, enhance saltiness and reduce off-flavors (Matlock, 1983). The use of phosphates may improve sensory and visual properties in restructured steaks and result in a product which resembles intact muscle in textural properties. Sodium tripolyphosphate (STP) has been studied (Puolanne and Terrell, 1983; Matlock, 1983) but has not been compared to sodium acid pyrophosphate (SAPP) or sodium hexametaphosphate (SHMP) for its effect on chemical traits, sensory characteristics, storage life or visual properties of restructured beef steaks. The objective of this study was to determine the effects of three phosphates (STP, SHMP, SAPP) on chemical, sensory, visual traits and storage life of restructured beef steaks formulated from lean bullocks.

MATERIALS & METHODS

SEVEN BLENDS (two replications each, 14 blends 9.1 kg raw meat) were formulated into restructured beef steaks at the Roman L. Hruska U.S. Meat Animal Research Center (Clay Center, NE). Beef lean was obtained from the rounds and chucks of bull carcasses and frozen at −35°C. The beef lean was segmented into strips (3 × 3 × 30 cm) on a band saw, tempered to −5°C and flaked by an Urschel Comitrol (Model 3600). Flaked and formed treatments were mixed in a paddle mixer for 20 min with additions of 0.75% NaCl and 0.25% or 0.5% of sodium tripolyphosphate (STP), sodium hexametaphosphate (SHMP) which is "sodium polyphosphates, glassy" by FDA-USDA nomenclature or sodium acid pyrophosphate (SAPP). All chemicals (salt and phosphates) were 99% pure reagent grade chemicals purphased from Fisher Scientific Company. All blends were stuffed into 16-cm cellulose casings and frozen to an internal temperature of −20°C. The logs were tempered to −5°C, pressed at 28 kg/cm^2 in a Ross press (Model 720), refrozen and cut into 2.5-cm steaks.

Three steaks from each treatment were cooked to an internal temperature of 70°C on Farberware Open Hearth broilers. Internal temperature was monitored by copper/iron thermocouple leads placed in the geometric center of each steak. Cooking time, thaw and cooking losses were determined for all steaks. After being cooked, each steak was visually scored for internal degree of doneness (6 = very rare, 1 = very well done) using photographic standards supplied by the National Live Stock and Meat Board. Sensory panel members (10) were trained according to Cross et al. (1978). Each member independently evaluated each sample for juiciness, tenderness, saltiness, bite, cohesiveness, flavor desirability, overall satisfaction and defects. The scale used was 8 = extremely juicy, tender, no salt, firm, cohesive, desirable or none and 1 = extremely dry, tough, salty, soft, crumbly, none, respectively. Restructured steaks were scored for defects, connective tissue, chunkiness, crumbly, coarse or off-flavor.

Three restructured steaks from each treatment were objectively measured for color using a D-25 Hunter Colorimeter after being thawed at 3°C for 24 hr. A five-member trained visual panel evaluated each blend for raw and cooked lean color (8 = extremely cherry red for raw and light brown for cooked; 1 = extremely dark brown for raw and cooked) and overall desirability (8 = extremely desirable; 1 = extremely undesirable) according to Jeremiah and Greer (1982). Steaks were displayed in a Tyler (Model DGC6) retail case under Sylvania Gro-Lux F4040W fluorescent lighting at 80 foot candles following commercial lighting conditions.

Moisture (raw) was determined using the vacuum oven procedure, fat by ether extraction and protein by Kjeldahl (AOAC, 1980). The percentage of Ca, Mg, K and Na were determined on each formulation by atomic absorption spectroscopy using a Perkin-Elmer® (model number 2300) machine. The samples were ashed (AOAC, 1980) and the proper dilutions were made in double distilled water to give readings in the proper absorption spectra. Thiobarbituric acid (TBA) analysis (Tarladgis et al., 1960) for the measurement of rancidity in lipids was conducted to determine storage life and the rate of oxidative rancidity development. TBA analysis was conducted to determine the development of oxidative rancidity after 10, 50, 70, 90, and 120 days at −20°C storage.

Authors Davis, Ramsey and Rolan are with the Meats Laboratory, Animal Science Dept., College of Agricultural Sciences, Texas Tech Univ., Lubbock, TX 79409. Author Miller is with Kleberg Center, Meats & Muscle Biology Section, Dept. of Animal Science, Texas A&M Univ., College Station, TX 77843. Author Seideman is with the Roman L. Hruska, U.S. Meat Animal Research Center, ARS, P. O. Box 166, Clay Center, NE 68933. Address inquiries to M.F. Miller.

Table 1—Means score for visual appearance and composition of restructured steaks containing two levels of three phosphates

	Type and level of (%) phosphate							
	Control	STP[a]		SHMP[a]		SAPP[a]		
Visual trait[b]	0	0.25	0.5	0.25	0.5	0.25	0.5	CV[c]
Raw lean color	3.7[g]	5.3[f]	6.5[d]	5.1[f]	5.9[e]	4.9[f]	3.3[g]	2.4
Raw overall desirability	4.1[f]	4.9[f]	6.9[d]	5.2[ef]	6.6[d]	5.5[e]	3.0[g]	2.7
Cooked lean color	5.2[d]	4.4[e]	4.1[e]	4.1[e]	3.7[f]	4.3[e]	5.5[d]	3.5
Cooked overall desirability	5.2[e]	5.5[de]	5.7[d]	5.3[e]	5.2[e]	3.4[g]	4.7[f]	3.2
Hunter color A	9.8[d]	9.0[e]	9.2[de]	8.0[f]	8.0[f]	9.3[de]	6.5[g]	3.6

[a]STP = sodium tripolyphosphate; SHMP = sodium hexametaphosphate; SAPP = sodium acid pyrophosphate.
[b] 3 = moderately brown, 6 = moderately bright cherry red; 3 = moderately undesirable, 6 = moderately desirable; 3 = moderately dark brown, 5 = slightly light brown; 3 = moderately undesirable, 5 = slightly desirable; Hunter color A = red.
c CV = coefficient of variation.
[d,e,f,g] Means in a row with a common superscript are not different (P>0.05).

Table 2—Means for certain chemical and mineral measures of restructured steaks containing two levels of three phosphates

	Type and level of (%) phosphate							
	Control	STP[a]		SHMP[a]		SAPP[a]		
Chemical measures	0	0.25	0.5	0.25	0.5	0.25	0.5	CV[c]
Moisture, %	65.6[d]	70.8[c]	69.9[c]	69.8[c]	69.6[c]	69.9[c]	70.4[c]	0.8
Fat (WTB)[g], %	14.2[c]	14.3[c]	14.5[c]	13.5[c]	13.6[c]	13.7[c]	13.9[c]	1.4
pH	5.9[d]	6.1[cd]	6.3[c]	6.0[d]	6.0[d]	5.9[d]	5.9[d]	1.5
Ca, %	0.0[d]	0.1[c]	0.1[c]	0.1[c]	0.1[c]	0.1[c]	0.1[c]	8.6
Na, %	0.2[f]	2.8[d]	3.5[c]	0.8[e]	3.1[cd]	1.9[e]	2.1[e]	46.2
K, %	1.2[e]	1.8[d]	2.9[c]	1.0[e]	2.0[d]	0.9[e]	1.1[e]	2.8
Mg, %	0.1[c]	0.0[d]	0.0[d]	0.1[d]	0.0[d]	0.0[d]	0.0[d]	82.7

[a] STP = sodium tripolyphosphate; SHMP = sodium hexametaphosphate; SAPP = sodium acid pyrophsphate.
[b] CV = coefficient variation.
[c,d,e,f] Means in a row with a common superscript are not different (P>0.05).
[g] WTB = whole tissue basis.

Table 3—Means for sensory traits of restructured steaks containing two levels of three phosphates

	Type and level of (%) phosphate							
	Control	STP[a]		SHMP[a]		SAPP[a]		
Sensory trait[b]	0	0.25	0.5	0.25	0.5	0.25	0.5	CV[c]
Bite	6.8[d]	5.2[e]	5.8[e]	5.8[e]	5.5[e]	5.7[e]	5.2[e]	12.3
Juiciness	6.9[d]	6.3[de]	5.7[e]	6.1[de]	6.5[de]	6.3[de]	5.7[e]	8.2
Tenderness	6.1[de]	5.8[e]	5.8[e]	5.6[e]	6.4[d]	5.7[e]	5.8[e]	5.7
Saltiness	5.8[d]	6.0[d]	6.0[d]	6.0[d]	5.8[d]	6.4[d]	6.1[d]	8.1
Cohesiveness	5.2[e]	5.5[de]	5.7[de]	6.1[d]	5.8[de]	5.8[de]	5.6[de]	8.7
Flavor desirability	6.7[d]	5.4[f]	6.2[de]	6.0[e]	5.3[f]	5.7[df]	5.7[ef]	6.4
Overall satisfaction	6.5[d]	6.4[d]	5.5[e]	5.7[e]	5.5[e]	5.5[e]	5.5[e]	7.6

[a] STP = sodium tripolyphosphate; SHMP = sodium hexametaphosphate; SAPP = sodium acid pyrophosphate.
[b] 5 = slightly firm, 6 = moderately firm; 5 = slightly juicy, 6 = moderately juicy; 5 = slightly tender, 6 = moderately tender; 5 = slightly salty, 6 = traces salt; 5 = slightly cohesive, 6 = moderately cohesive; 5 = slightly desirable, 6 = moderately desirable; 5 = slightly desirable, 6 = moderately desirabe.
[c] CV = coefficient of variation.
[d,e,f] Means in a row with a common superscript are not different (P>0.05).

Table 4—Textural properties of restructured steaks containing two levels of three phosphates

	Type and level of (%) phosphate							
	Control	STP[a]		SHMP[a]		SAPP[a]		
Textural traits[b]	0	0.25	0.5	0.25	0.5	0.25	0.5	CV[c]
Peak load	4.5[e]	5.8[d]	5.9[d]	3.8[f]	4.2[ef]	4.4[e]	4.7[e]	8.3
Fail energy	12.0[de]	14.0[d]	12.4[d]	8.2[f]	10.8[e]	10.8[e]	11.7[e]	13.9
Fail elongation	5.8[d]	5.4[d]	5.3[d]	5.5[d]	5.5[d]	5.6[d]	5.7[d]	5.0

[a] STP = sodium tripolyphosphate; SHMP = sodium hexametaphosphate; SAPP = sodium acid pyrophosphate.
[b] Peak load, expressed as kg-force, maximum force required to shear sample; fail energy expressed as cm-kg-force, index of amount of work required to shear sample; fail elongation expressed as cm-kg-force, index of distance required to shear a sample.
[c] CV = coefficient of variation.
[d,e,f] Means in a row with a common superscript are not different (P>0.05).

Steaks were cooked as described previously for the sensory analysis and sampled for textural properties. After a 2 hr cooling period, six 1 × 1 × 1 cm cube sections were removed from each of three steaks. The textural variables of the test samples were measured by the Universal Instron Testing Machine (Model 11321) Microcon II. The fail criterion was set at 75%, the chart paper speed at 20 cm/min and the crosshead speed at 5 cm/min. Peak energy, peak load, peak elongation, fail energy, fail load and fail elongation were determined on six 1 × 1 × 1 cm cubes per sample. Because the data analysis revealed few differences among the six textural parameters, only peak load, fail energy and fail elongation data will be presented.

An analysis of variance (Steel and Torrie, 1980) for a 7 (blend) × 3 (level) × 2 (replication) factorial design and appropriate interactions was used to analyze all traits according to SAS (1982). Type and level of phosphate were used as the main effects to analyze the data. When the main effect was significant, mean separation was accomplished using the Bonferroni's test (Kirk, 1982). The predetermined acceptable level of probability was 5% for all analyses and was used throughout this discussion.

RESULTS & DISCUSSION

NO SIGNIFICANT INTERACTIONS occurred between blends or level. Data for visual properties of raw restructured steaks (Table 1) indicated five of the six blends formulated with 0.25% or 0.5% STP, SHMP or SAPP had a more desirable (P<0.05), darker, cherry red color than the control steaks. However,

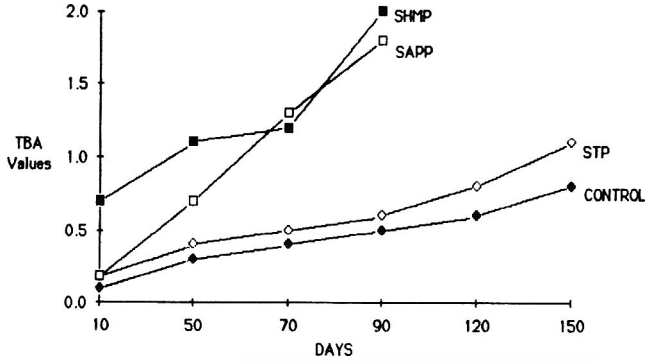

Fig. 1—Thiobarbituric acid analysis for phosphates and control restructured steaks.

steaks made with 0.5% SAPP were brown in raw color and had undesirable raw color scores. These results may be due to the loss of water in products formulated with SAPP. The loss of water between the intermolecular meat pieces may cause an accelerated formation of metmyoglobin resulting in a darker brown color. Additionally, the loss of water may be due to a lower pH which causes the meat to be closer to the isoelectric point of the meat proteins causing them to lose their ability to bind water. Among the five treatments acceptable in overall raw desirability, steaks made with 0.5% STP, 0.5% SHMP and 0.25% SAPP were scored higher ($P<0.05$) than the control. These data indicated raw color and desirability advantages may exist for restructured steaks formulated with either STP, SHMP or 0.25% SAPP.

Cooked lean color of restructured steaks formulated with 0.5% SAPP and the control was not different ($P>0.05$) and was lighter brown in color. However, the blend formulated with 0.5% SHMP was rated darker in cooked lean color. Control and restructured steaks formulated with STP and SHMP were higher in cooked desirability scores than steaks made with SAPP, indicating that if restructured steaks were sold as precooked items in transparent packages, the use of STP and SHMP as added phosphates might be beneficial to final consumer acceptability. Additionally, restructured steaks formulated with SAPP were rated slightly to moderately undesirable for cooked appearance and may not be feasible to use in precooked, restructured steaks. The data in Table 1 indicate that 0.5% STP would be the best phosphate to use in restructured steaks to maximize the raw lean color and raw or cooked desirability of these restructured steaks.

All raw phosphate-treated steaks contained more moisture ($P<0.05$) than the control (Table 2). As expected, the addtion of phosphates to restructured steaks improved moisture retention due to the increased extraction of muscle proteins that increased availability of charged groups available to bind more water in the meat matrix. Fat content of the steaks did not show significant ($P>0.05$) differences because an effort was made to consistently formulate the blends. Sodium tripolyphosphate at the 0.5% level caused a significant increase in the pH of restructured steaks when compared to the control, SHMP and SAPP formulations. The increase in pH by 0.5% STP was confirmed by rancidity values since the increase in pH increased the storage life of the STP added restructured steaks. Additions of phosphates increased ($P<0.05$) the percentage of calcium and sodium when compared to the control formulation. The calcium may have been leached out by the addition of these phosphates or the increased level of phosphate may have interferred with the Ca analysis. Although sodium values varied from 0.21% to 3.08%, the sensory panel did not detect these differences. All phosphate added formulations contained significantly more calcium and sodium than the control. The control contained more ($P<0.05$) magnesium than all phosphate added restructured steaks, but treatments did not affect magnesium levels among phosphate. These results for Ca, K and Mg appear to be negligible since the added phosphates may interfere with these readings and cause the accuracy to decline.

In data not presented in tabular form, thaw loss, cooking loss and degree of doneness scores were not affected by phosphate type or level. Thaw losses were less than 1.0% and indicated that little loss of moisture or weight occurred during thawing.

Restructured steaks containing phosphates had lower ($P<0.05$) bite scores, indicating less resistance when compared to the control steaks (Table 3). All sensory traits for all treatments were scored in the "slightly to moderately" range by the panel. The control contained less moisture (Table 1) and was rated significantly more juicy (Table 3) than blends containing 0.5% STP or SAPP. Blends containing 0.5% SHMP were more tender than other phosphate added steaks but did not differ from the control. However, tenderness differences among restructured steaks were probably minimal, since all lean and fat sources were flaked prior to the formulation of each blend and mixing procedures were the same for all blends.

Saltiness ratings were not different among all formulations indicating added phosphates had no interaction effect on perceived saltiness of the final product. Sensory panel cohesiveness ratings were significantly higher ($P<0.05$) for restructured steaks with added phosphates compared to control steaks suggesting that phosphates assisted in the binding of meat particles. Steaks from blends containing 0.5% STP and 0.25% SAPP did not differ from the control in flavor desirability ratings. Although slightly to moderately desirable in flavor, steaks from the other four treatments were less desirable than the control steaks. The control and 0.25% STP also were not different ($P>0.05$) for overall desirability scores. All other phosphate formulations were less desirable overall than the control.

Textural properties of shear force (peak load), work required to shear the sample (fail energy) and distance required to shear the sample (fail elongation) are shown in Table 4. Peak load values indicated that the addition of phosphates increased ($P<0.05$) the amount of force required to shear the product indicating that the addition of phosphates will improve the binding of meat pieces of restructured steaks. No significant ($P>0.05$) differences were found for fail elongation since all steaks were precut to the same thickness prior to cooking. Steaks made with STP required more ($P<0.05$) force and work to shear than all other restructured steaks formulated with phosphates, which would indicate an increase in binding or greater cohesiveness. These data agree with Puolanne and Terrell (1983) who reported firmness scores increased in frankfurters formulated with STP.

Data on sensory defects, not presented in tabular form, indicated that all phosphate formulated steaks had some type of off-flavor and saltiness of the final product. Sensory panel cohesiveness ratings were significantly higher ($P<0.05$) for restructured steaks with added phosphates compared to control steaks suggesting that phosphates assisted in the binding of meat particles. Steaks from blends contained some detectable connective tissue. These findings agree with Karmas (1970) and Ellinger (1972) who also found off-flavors increased with added phosphates. The use of bull forequarters as the lean source and the change in pH may have contributed to the connective tissue and off-flavor defect problems. Breidenstein (1982) also reported that the increased connective tissue content of restructured steaks decreased their desirability. Formulation had no effect on fineness defects. All but four blends were found to be coarse, crumbly or chunky which could be due to the high amount of connective tissue in each steak.

Thiobarbituric acid analysis showed that steaks with added STP had acceptable (less than 1.0) TBA values until after 120 days of frozen storage (Fig. 1). Thus, restructured steaks made with STP have a distinct advantage in storage life compared to steaks made with SHMP or SAPP because steaks formulated with SHMP and SAPP were rancid by 70 days frozen storage.

These TBA values indicated that restructured steaks made with STP had slower oxidation of fats and were superior in frozen storge when compared to steaks formulated with either SHMP or SAPP. STP was able to chelate metal ions and increased the pH as reported previously. The STP increased the pH of meat batters and may have slowed the rate of oxidation. Also, the ability of phosphates to work as metal chelates will slow the formation of peroxidation products. The SAPP decreased the pH of the meat batters and the decrease in pH of meat will increase the rate of lipid oxidation. The poor chelating of the acid phosphate also may have played a very significant role in increasing the rate of oxidation. The SHMP phosphates also increased the rate of oxidative rancidity. The increase in free radical formation as a result of increased numbers of phosphate groups may have resulted in the increased rate of oxidation in SHMP blends.

CONCLUSIONS

DATA from the present study indicated restructured steaks formulated with 0.25% or 0.5% STP were generally rated higher for palatability scores, visual properties, textural properties and had a longer storage life than control or other phosphate added restructured steaks. Restructured steaks formulated with SHMP were "slightly desirable" for sensory overall desirability scores, had improved raw and cooked appearance ratings and had increased binding properties compared to the control. However, these steaks were rancid after 90 days of freezer storage and panelists found off-flavors in 25% of the samples. Blends made with SAPP were acceptable for sensory traits and binding properties but were undesirable in visual properties, storage life and had off-flavors in 40% of all sensory panel member evaluations. Therefore, STP or SHMP will improve raw and cooked appearance, binding properties and still produce acceptable sensory traits of restructured steaks. The use of STP or SHMP in restructured beef steaks would be of merit to the meat industry.

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Effect of Salt Reduction on the Yield, Breaking Force, and Sensory Characteristics of Emulsion-Coated Chunked and Formed Ham

LESLIE F. THIEL, PETER J. BECHTEL, FLOYD K. McKEITH, JAN NOVAKOFSKI and TOM R. CARR

ABSTRACT

The effect of a modified processing procedure (emulsion coating) on the characteristics of ham with reduced sodium chloride (salt) content was investigated. Emulsion coated (EC) and conventionally processed (CP) chunked and formed hams were prepared with 0.5, 1 and 2% added salt. When compared to CP hams, EC hams had increased cooking yield, breaking force, moisture retention, juiciness, texture, tenderness, and taste panel acceptability. The EC 1.0% salt treatment closely resembled the CP 2.0% salt treatment. The EC 0.5% salt product had many similarities to the CP 1.0% salt treatment but was significantly different in many attributes from the CP 2% salt product. Reducing salt in chunked and formed ham from 2.0% added salt addition to levels as low as 1.0% appears possible when combined with emulsion coating to minimize the effects of salt reduction.

INTRODUCTION

THE ROLE of dietary sodium in human health and the implications of sodium reduction in muscle food has been reviewed by Sebranek et al. (1983). A 1982 survey by General Foods ranked sodium content of food at the highest level of consumer concern (Lovrien, 1982). Awareness of the sodium content in processed meats has led to an ever increasing interest in reducing salt content in these products (Abernethy, 1982; IFT, 1980; Lovrien, 1982; Terrell and Olson, 1981; Wilson, 1982). Terrell (1983) has reviewed the effect of reduced sodium in a number of processed meat products. Questions concerning the consequences of reducing the salt level in processed meats need to be addressed before low salt processed items can be produced successfully.

Salt provides flavoring properties to meat and also alters functional characteristics such as water-holding capacity and product binding. Water binding to muscle proteins is dramatically increased by salt at comparable water activities (Lioutas et al., 1984). Reducing the salt content of processed meats can also adversely affect the value and yield of salable product (Hamm, 1960; Ford et al., 1978; Theno et al., 1978a, b; Terrell and Olson, 1981). Thus, alternate processing technologies are being sought to compensate for changes resulting from decreases in the level of salt. It has been demonstrated that the functional advantages associated with tumbling or massaging of ham products is accompanied by an increased extraction of proteins (Motycka and Bechtel, 1983) which is enhanced by salt (Siegel et al., 1978 a,b). Various proteins have been added to restructured products in order to test their effect on binding (Siegel and Schmidt, 1979; Ford et al., 1978; Macfarlane et al., 1977; Froning, 1966; Fukazawa et al., 1961; Moore et al., 1976). Although many proteins including the myofibrillar proteins showed favorable results, many are not readily available for commercial processing nor are they cost effective. Tsai and Ockerman (1982) used an alternate approach in which they made a meat emulsion and added it to a restructured pork product. Their results indicated that emulsion coating of meat aggregates mimicked tumbling by increasing yield and sensory properties and resulted in acceptable binding characteristics.

A recent study from our laboratory examined the effect of reducing salt on the sensory and processing properties of chunked and formed ham (Thiel et al., 1986). Hams were made with 0, 0.5, 1, 1.5, and 2% added salt. Results indicated that the 1.5% salt treatment possessed characteristics closely resembling the 2.0% salt treatment; however, the 1.5% salt treatment had a slight reduction in yield, breaking force, and visual acceptability. The addition of 0.5 and 1.0% salt resulted in significantly lower yields and less desirable sensory characteristics. We postulated that further reduction of salt in chunked and formed ham was possible using emulsion coating technology. No evaluation has been made of adding a meat emulsion to a reduced salt product. The objective of this experiment was to examine the effect of reducing added salt in an emulsion-coated, chunked, and formed ham on the yield, breaking force, visual, and sensory characteristics of the final product.

MATERIALS & METHODS

Fresh meat preparation

Approximately 55 kg fresh, uncured, boneless ham was diced into 2–3 cm cubes. The chunks were prepared from closely trimmed semimembranosus, adductor, biceps femoris, and semitendinosus muscles from market hogs of similar weight and genetic background. The hogs had been slaughtered 2–3 days prior to muscle separation. All ham chunks were mixed for two min to randomize animal and muscle variation. Six aliquots (7 kg each) of ham chunks were placed in sealed plastic bags and stored at 4°C for up to 20 hr before further processing.

Curing solution preparation

Curing solutions were formulated to result in products that would contain 0.5% of a sodium tripolyphosphate and sodium hexametaphosphate blend (Stauffer's Cur-A-Phos 11-2), 0.33% dextrose, 550 ppm sodium erythorbate, and 120 ppm sodium nitrite after cooking. The curing solutions also contained sufficient sodium chloride to result in 0.5%, 1.0%, and 2.0% salt added as a percent of the green, uncured ham weight.

Ham and emulsion preparation

Two 7 kg aliquots of fresh ham were placed in a Universal 190 Inject Star Tumbler (Globus Labs, Hackensack, NJ) with curing solution and then tumbled for 4 hr at 28 revolutions per min with vacuum (13–21 kPa). The product was removed from the tumbler; a 7 kg aliquot was used for the nonemulsion coating treatment and a 6.3 kg aliquot for the emulsion coating treatment. Separate emulsions were prepared for each treatment by chopping a mixture consisting of 5 kg uncured ham, 100g salt, 25g phosphate blend, 16.7g dextrose, 2.8g sodium erythorbate, 0.6g sodium nitrite, and 605 g ice in a bowl chopper equipped with six knives for approximately 10 min. Seven hundred grams of the emulsion were mixed by hand with the 6.3 kg tumbled ham aliquot for 2 min. The 7 kg nonemulsion-coating treatment was also mixed by hand for 2 min.

The ham emulsion added to all treatments contained 1.7% sodium chloride. The sodium chloride content of the emulsion was not varied to avoid differences in emulsion composition. This results in the emulsion-coated treatments having 0.62, 1.07, and 1.97% added sodium

The authors are with the Dept. of Animal Sciences, 205 Meat Science Lab., University of Illinois, 1503 S. Maryland Drive, Urbana, IL 61801-4737. Direct inquiries to Dr. Bechtel. Author Thiel's current address is Dept. of Animal Science, Cornell Univ., Ithaca, NY 14850.

Table 1—Yields, Instron breaking force and composition of conventionally processed (CP) and emulsion coated (EC) chunked and formed hams

	Treatment					
	0.5% Salt		1.0% Salt		2.0% Salt	
	CP	EC	CP	EC	CP	EC
Precooked yield, %	98.3^{ab}	98.5^{a}	98.0^{b}	98.2^{ab}	98.5^{ab}	98.4^{ab}
Cooked yield, %	84.3^{a}	89.7^{b}	91.6^{b}	94.4^{c}	95.4^{c}	96.0^{c}
24 hour storage yield, %	82.7^{e}	88.2^{b}	90.3^{b}	92.6^{c}	94.2^{c}	94.7^{c}
Free water, %	58.2^{z}	55.0^{ab}	51.9^{b}	50.9^{b}	52.9^{b}	46.5^{c}
Moisture, %	72.1^{a}	72.3^{a}	72.8^{abc}	73.3^{bc}	72.6^{ab}	73.6^{c}
Fat, %	5.3^{a}	5.3^{a}	4.9^{ab}	4.6^{ab}	5.1^{ab}	4.1^{b}
Instron breaking force, g/cm^2	96.2^{a}	115.5^{ab}	124.4^{b}	153.9^{c}	215.2^{d}	240.7^{e}

$^{a-e}$ Different superscripts within a row indicate significant differences among means ($P<0.05$).

Table 2—Sensory evaluation of conventionally processed (CP) and emulsion coated (EC) chunked and formed hams

	Treatment					
	0.5% Salt		1.0% Salt		2.0% Salt	
	CP	EC	CP	EC	CP	EC
Visual binding	51.6^{a}	69.3^{ab}	86.9^{bc}	98.3^{c}	111.8^{c}	111.0^{c}
Cured color intensity	53.9^{a}	51.2^{a}	67.2^{a}	59.9^{a}	89.8^{b}	89.2^{b}
Cured color uniformity	56.6^{a}	67.7^{ab}	63.9^{ab}	89.2^{b}	83.0^{ab}	80.6^{ab}
Visual acceptability	38.0^{a}	49.9^{ab}	72.0^{bc}	78.8^{c}	94.2^{c}	95.2^{c}
Saltiness	41.5^{a}	47.3^{ab}	55.9^{bc}	63.0^{c}	76.1^{d}	77.9^{d}
Juiciness	70.2^{a}	82.4^{b}	78.4^{ab}	80.2^{b}	81.9^{b}	83.8^{b}
Texture	64.8^{a}	71.2^{ab}	79.0^{bc}	86.0^{cd}	88.0^{cd}	93.0^{d}
Tenderness	80.2^{a}	91.0^{a}	84.7^{a}	86.7^{a}	82.0^{a}	89.5^{a}
Taste acceptability	61.4^{a}	68.7^{a}	81.5^{b}	86.7^{b}	90.4^{bc}	97.3^{c}

$^{a-d}$ Different superscripts within a row indicate significant differences among means ($P<0.05$).

chloride which were compared to the 0.5, 1.0, and 2% sodium chloride conventionally processed treatments.

A hydraulic stuffer was used to stuff each treatment into four separate chubs (Teepak 4s fibrous casings) and the chubs were held 24 hr at 4°C prior to cooking. A thermocouple was placed in the center of each chub which was then placed in a cooking bag and cooked in a hot water bath maintained at 80–85°C for approximately 1 hr. The hams were removed when an internal temperature of 68°C was reached. They were then immersed in a 0°C ice water bath for 30 min and subsequently held in a 4°C cooler. The weight of each chub was recorded after stuffing (stuffed weight), immediately prior to cooking (precook weight), after the ice water chill (cooked weight) and after 24 hr (24 hr weight) storage in a 4°C cooler. Yields were determined as a percent of stuffed weight.

Fifteen 1 cm slices were cut from each chub for analysis. The first two slices were discarded. The next ten slices were randomly assigned as follows: five slices for breaking force determination, two slices for proximate analysis, and three slices for sensory panel evaluation. The slices were vacuum packaged and held at 4°C in closed, cardboard boxes until analyzed.

Breaking force determinations

An Instron Universal Testing Machine Model 1132 equipped with a 50 kg load cell and using a chart and crosshead speeds of 20 cm/min was used to determine the breaking force by the method of Siegel et al. (1978b). Each of five slices was placed on the bridge adjusted to a width of 38.7 mm and broken by a 6.5 mm diameter bar mounted in the Instron chuck. The peak force required to rupture the slice was recorded and divided by the cross sectional area of the slice in order to determine maximum force per cross sectional area (g/cm^2) required to break the slice.

Free water, moisture and fat determinations

Water-holding capacity was determined by the method of Wierbicki and Deatherage (1958). Approximately 500 mg of the sample was weighed onto a 9 cm diameter Whatman Number 1 filter paper. A sheet of acetate was placed above the sample and the sample was then pressed at 35 kg/cm^2 in a Carver Laboratory Press for 1 min and percent free water determined from the area of meat and exudate on the filter paper. Percent moisture was determined by the air oven method described by Koniecko (1979). Percent fat determination was accomplished by repetitive chloroform:methanol (2:1) extraction of the dried samples obtained after the moisture analysis (Riss et al., 1983).

Visual and palatability evaluation

Visual evaluation included appraisal of binding characteristics, cured color intensity, cured color uniformity, and visual acceptability as a ham product. The palatability attributes evaluated included saltiness, juiciness, texture, tenderness, and taste acceptability as a ham product. Six experienced sensory panel members recorded their evaluation of each characteristic by placing a hash mark on a 14 cm line anchored at each end with words representing extremes of the characteristics. The anchor terms (from the left mark) were: binding—very loosely bound to very tightly bound; visual color intensity—very pale to very dark; visual color uniformity—very nonuniform to very uniform; visual acceptability as a ham product—very unacceptable to very acceptable; saltiness—very bland to very salty; juiciness—very dry to very juicy; texture—very undesirable to very desirable; tenderness—very tough to very tender; and taste acceptability as a ham product—very unacceptable to very acceptable. Sensory panel members were familiarized with the ballot using products representing various points on the scale. Scoring was evaluated by measuring the distance from the left anchor mark to the panelist's hash mark in millimeters.

Panelists visually evaluated one slice of each ham chub five days after ham preparation. The slices were randomly selected from the three slices assigned to sensory evaluation. The visual appraisal was performed at 23°C under cool white fluorescent lighting with slices presented on a white background.

One slice was randomly selected from each chub and cut into wedges for palatability evaluation five days after manufacture. Samples were individually presented to the panelists at 23°C under red lighting. Eight samples were served to each of the six member panel at 9:00 a.m., 11:00 a.m., and 1:30 p.m.

Statistical analysis

Results presented in Tables 1 and 2 were analyzed using the Statistical Analysis System (SAS, 1982) procedure for analysis of variance, and Duncan's multiple range test for separation of means. The t-test was used to analyze pooled values from the three salt treatments in Tables 3 and 4.

RESULTS & DISCUSSION

Yield, moisture and fat analysis

The yield data are shown in Table 1. The precooked yield was unaffected by salt addition or emulsion coating, except that the 1.0% salt addition with no emulsion-coating treatment yielded significantly less product than the 0.5% salt addition with emulsion-coating. The ranked order for cooked yield was

Table 3—Effects of conventional processing (CP) and emulsion-coating (EC) on yields, Instron breaking force and composition of chunked and formed hams

	CP	EC
Precooked yield, %	98.3[a]	98.4[a]
Cooked yield, %	90.4[a]	93.4[b]
24 hour storage yield, %	89.1[a]	91.9[b]
Free water, %	54.3[a]	50.8[b]
Moisture, %	72.5[a]	73.1[b]
Fat, %	5.1[a]	4.7[a]
Instron breaking force, g/cm^2	145.3[a]	170.0[b]

[a,b] Different superscripts within a row indicate significant differences among means ($P<0.05$).

Table 4—Sensory evaluation of conventionally processed (CP) and emulsion coated (EC) chunked and formed hams

	CP	EC
Visual binding	83.4[a]	92.9[a]
Cured color intensity	70.3[a]	66.8[a]
Cured color uniformity	67.8[a]	79.2[a]
Visual acceptability	68.1[a]	74.7[a]
Saltiness	57.8[a]	62.8[b]
Juiciness	76.8[a]	82.2[b]
Texture	77.3[a]	83.4[b]
Tenderness	82.3[a]	89.1[b]
Taste acceptability	77.8[a]	84.2[b]

[a,b] Different superscripts within a row indicate significant differences among means ($P<0.05$).

2.0% salt with emulsion-coating (2.0% EC) = 2.0% salt without emulsion coating (2.0% CP) = 1.0% EC > 1.0% CP = 0.5% EC > 0.5% CP. Storage yields showed no significant differences independent of cooking yield ($P>0.05$).

The percentage of free water was highest for 0.5% CP and lowest for 2.0% EC ($P<0.05$) (Table 1). A trend was noted for the EC products, where increased salt level decreased the percentage of free water. As expected, fat content varied little (5.3 to 4.1%); however, some significant differences were observed.

Instron breaking force

Analysis of the Instron breaking force values (Table 1) indicated 2.0% EC > 2.0% CP > 1.0% EC > 1.0% CP ($P<0.05$). The mean breaking force for 1.0% CP was not different from 0.5% EC but was greater than 0.5% CP. Emulsion-coating increased breaking force at the 1.0% and 2.0% salt level. Increased salt also increased breaking force, which agrees with the results of Maesso et al. (1970), Moore et al. (1976), Nakayama and Sato (1971), and Siegel et al. (1978b). Trout and Schmidt (1984) examined the effects of adding phosphate and salt on the binding of restructured beef rolls and found changes in binding could be explained in terms of pH and ionic strength.

The increase in breaking force resulting from emulsion-coating (Tables 1 and 3) is in agreement with the observations of Tsai and Ockerman (1982) who found that emulsion-coating increased breaking force (binding) at the 3% salt level in the same manner as tumbling did. Theno et al. (1978a, b) have shown tumbling of meat results in an emulsion-like exudate in the binding junctures of restructured products.

Visual and palatability evaluations

The sensory evaluation data are shown in Tables 2 and 4. Differences in visual evaluation of binding characteristics were not detected between emulsion coated or uncoated treatments within a salt level. However, the 0.5% salt treatments were perceived as having less binding than 1.0% of 2.0% salt levels. Cured color intensity was most desirable for the 2.0% added salt treatments and the reason for the large increase over the 1% added salt treatment is not known. However, it is possible that salt benefits color intensity. Few differences in cured color uniformity were noted; although, there was a numerical tendency for the higher salt levels to be scored as more uniform. Overall visual acceptability of the products as ham ranked the 0.5% salt additions below the 1 and 2% salt treatments, except that 0.5% EC was not significantly different from 1.0% CP.

Palatability evaluation of saltiness indicated that higher salt levels resulted in higher sensory values ($P<0.05$); however, the 0.5% EC treatment was similar to 1.0% CP. Similarly, texture scores increased with increasing salt level. There were no differences in sensory tenderness scores ($P>0.05$). Overall taste panel acceptability of the products as ham indicated significant increases in acceptability with higher salt additions; however, the 1.0% EC was not different from 2.0% CP.

Effects of emulsion-coating

Table 3 shows the effect of emulsion-coating on yield characteristics. The major difference found was that emulsion-coating substantially increased the cooked yield ($P<0.05$).

No difference was found in fat content of emulsion coated treatments versus noncoated treatments (Table 3). Furthermore, the emulsion coated treatments had significantly higher moisture content with a smaller portion of the water as free water ($P<0.05$). Emulsion-coating resulted in a substantial increase in the Instron breaking force ($P<0.05$). Also, no visual differences were detected as a result of emulsion-coating (Table 4). However, saltiness, juiciness, texture, tenderness, and overall taste panel acceptance of the product as ham were all improved by emulsion-coating ($P<0.05$). It is doubtful that the small differences in salt content between comparable emulsion-coated and conventionally processed treatments has substantially altered any comparisons between the 2% treatment and either the 1% or 0.5% treatment; however, effects between the 0.5% and 1% salt treatment warrants further evaluation. Tsai and Ockerman (1982) reported that emulsion-coated, sectioned and formed pork had superior flavor and textural appeal to tumbled samples. However, their tumbled samples had better cohesiveness scores, but emulsion-coated and tumbled samples had similar yields.

Emulsion-coating was an effective means to enhance cooking yield, breaking force, and the palatability scores for saltiness, juiciness, texture, tenderness, and taste acceptability. The emulsion-coated 1% salt product had many characteristics similar to the nonemulsion-coated 2% salt product. This suggests that, with emulsion-coating, salt could be reduced from 2% to 1% in chunked and formed ham products with minimal sacrifices in breaking force and cured color intensity. This could allow significant reduction in sodium content of chunked and formed ham. Many characteristics of the product containing 0.5% sodium chloride with emulsion-coating were improved over the 0.5% sodium chloride, conventionally processed product, although they were less desirable than values for conventionally processed ham containing 2% salt.

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Feasibility of Using Catalase Activity as an Index of Microbial Loads on Chicken Surfaces

GEORGE I. J. WANG and DANIEL Y.C. FUNG

ABSTRACT

The Catalasemeter with membrane filter method was tested to quantitate the catalase activity of laboratory cultures as well as swab samples from cold-stored (7°C) chicken as measurements of microbial activity. Catalasemeter reading (flotation time in sec) is inversely proportional to total number of catalase producing bacteria. Good correlation ($r = -0.93$) was obtained between Catalasemeter and both viable cell population and psychrotroph population of cold-stored chicken. For good quality cold-stored chicken ($<10^3$ CFU/cm^2), the flotation time was longer than 4,200 sec; and in the case of poor quality cold-stored chicken ($>10^6$ CFU/cm^2), the flotaton time was less than 580 sec. This provides a guideline for rapidly estimating microbial loads in cold-stored chicken.

INTRODUCTION

SEVERAL IMPORTANT BACTERIA in the environment can be divided into catalase-positive and catalase-negative groups. Because catalase is a constitutive enzyme of aerobic bacteria, the concentration of catalase increases as the number of bacteria increases, making it possible to use catalase activity to estimate bacterial concentration under certain conditions (Fung, 1985). The Catalasemeter test utilizes the disc-flotation method proposed by Gagnon et al. (1959). Charbonneau et al. (1975) used the Catalasemeter with paper disc system to detect and measure bacterial catalase activity of *Bacillus* spp. and *Enterobacteriaceae* and reported that different species had different catalase-producing ability. Dodds et al. (1983) applied the *Limulus* Amoebocyte Lysate test and Catalasemeter with paper disc system to the study of the microbial quality of vacuum-packed cooked turkey ham and reported that the Catalasemeter was not sensitive enough to detect bacterial loads of less than 10^4 CUF/g. However, Ayres (1955) reported that, although a great variety of organisms could be found on freshly slaughtered birds, a much smaller variety (several species of *Pseudomonas*) was responsible for the spoilage of refrigerated poultry meat. A preliminary study of laboratory cultures showed that *Pseudomonas* spp. are strong catalase producers. The purpose of this investigation was to determine the feasibility of using the Catalasemeter method to measure catalase activity as an index of microbial loads on chicken surfaces.

MATERIALS & METHODS

Catalasemeter

An instrument called the Catalasemeter was recently introduced commercially by Bio-Engineering Group Ltd. (New Haven, CT). Its use involves saturating a paper assay test disc with the test liquid (0.14 mL) and dropping the disc into a tube containing 5 mL of 3% H_2O_2 solution (with 10^{-6}M EDTA), which was previously placed in the Catalasemeter. If the test liquid contains catalase, gas bubbles will be generated and trapped beneath the paper disc, causing the disc to float. The time lapse in seconds between the disc sinking down and floating up is the flotation time (FT) of the disc. The flotation time is inversely proportional to the concentration of catalase in the solution absorbed by the disc.

The sensitivity of the Catalasemeter test is improved by using a 13 mm diameter, 0.45 μm pore size, membrane filter instead of an assay paper disc. A sample of liquid is first passed through the membrane filter, then the filter is dropped into the H_2O_2 solution in the Catalasemeter.

Study of purified catalase and bacterial catalase activity

To establish sensitivity of the Catalasemeter, the activities of purified catalase and bacterial cultures were tested. Commercially purified lyophilized *Aspergillus niger* catalase (Calbio Chem-Behring Co., San Diego, CA) was dissolved in 0.05M phosphate buffer at pH 7.0 and a series of dilutions was made to obtain catalase concentrations from 10^4 to 10^{-4} catalase units/mL. Six catalase-positive bacteria: *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 25922, *Micrococcus luteus* ATCC 4689, *Pseudomonas fluorescens* ATCC 13525, *Salmonella typhimurium* ATCC 23566, *Staphylococcus aureus* KSU 100; and two catalase-negative bacteria: *Lactobacillus brevis* ATCC 14869 and *Streptococcus faecalis* ATCC 19433 were used to test the accuracy and sensitivity of the Catalasemeter for detecting catalase-producing ability of bacterial cultures. Test cultures were prepared by aseptically transferring one loopful of culture from stock culture into 20 mL of Brain Heart Infusion Broth (BHI, Difco) and incubating the culture for 24 hr at each organism's optimal growth temperature. At the end of this incubation, broth culture was diluted, and split samples were evaluated for catalase activity by the Catalasemeter and for viable cell count by the standard plate count method (Clark et al., 1978) and reported as colony forming unit (CFU) per mL.

Study of catalase activity on cold-stored chicken

Chicken parts were purchased at a local supermarket and stored at 7°C. The swab technique was used for sampling. This technique involved wetting a sterile cotton swab (American Scientific Procedure. McGaw Park, IL) in a tube containing 2.5 mL of sterile pH 7.0 phosphate buffer. After swabbing the meat surface (2×4 cm^2) in three directions, the cotton swab was broken into the original tube aseptically and shaken vigorously 40 times. One milliliter of swab sample solution (nonacidified) was diluted for a comparative study of viable cell count (32°C, 48 hr) and modified psychrotroph count (21°C, 23–25 hr) according to the standard plate count procedure. Another 1 mL of swab sample solution was then acidified with pH 3.25 phosphate buffer to pH 3.30. The acidification procedure was found to be necessary to inhibit interfering catalase activities from blood contaminating chicken surfaces (Wang, 1985). After filtration, the membrane was applied to the Catalasemeter as previously described.

Statistical methods for analysis

A nonlinear model (SAS, 1982) was used to construct the equation between logarithm of flotation time (sec) and logarithm of colony forming units/filter using data generated from cold-stored chicken.

RESULTS & DISCUSSIONS

Purified catalase and bacterial catalase activities

Figure 1 shows the standard curve developed for catalase quantification by the Catalasemeter with the paper disc system. Two linear slopes were observed between the logarithm of the flotation time (FT) and the logarithm of the catalase concentration tested. The correlation coefficient of the first slope (10^4 to 10^0 catalase units/mL) was $r = -0.99$ ($P<0.01$) and the equation for the regression line was Log FT (sec) = 2.311 –

Authors Wang and Fung are with the Dept. of Animal Sciences & Industry, Kansas State Univ., Manhattan, KS 66506.

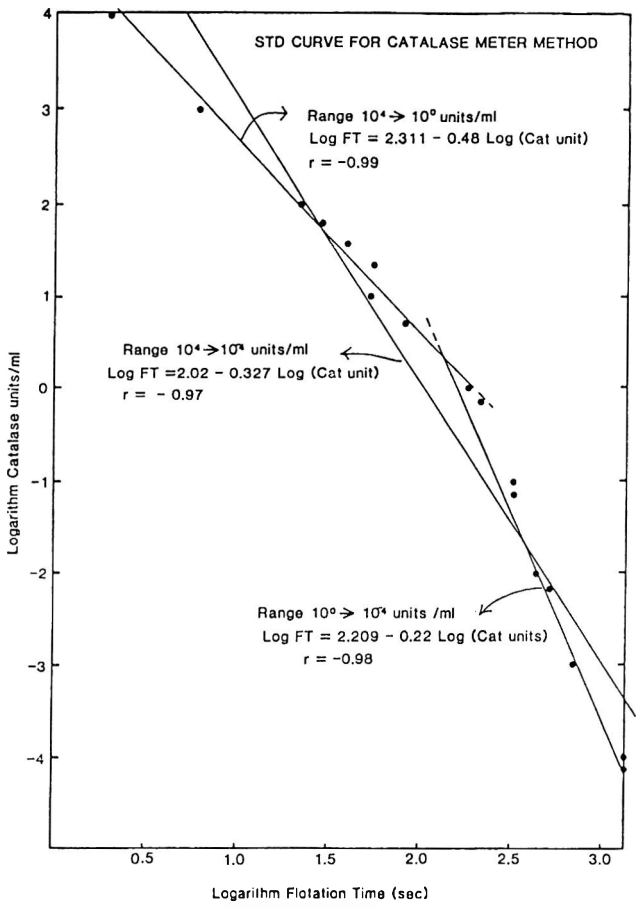

Fig. 1—Standard curve for catalasemeter with paper disc method on purified Aspergillus niger *catalase.*

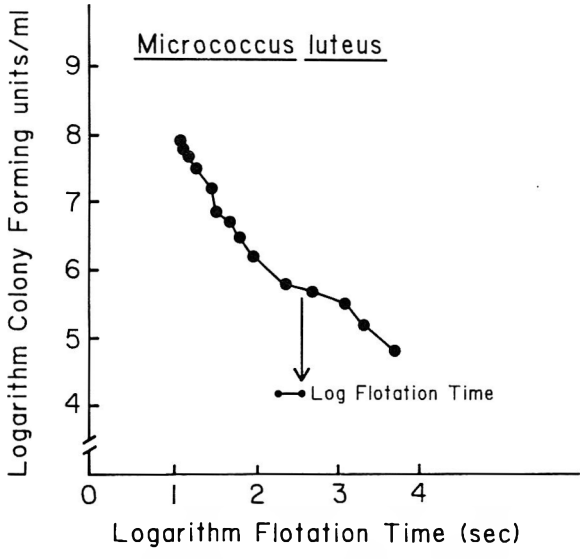

Fig. 2—Measurement of catalase activity of Micrococcus luteus *by Catalasemeter with paper disc method.*

0.48 log catalase units/mL. The correlation coefficient of the second slope (10^0 to 10^{-4} catalase units/mL) was r = −0.98 ($P<0.01$) and the equation for the regression line was log FT = 2.209 − 0.22 log catalase units/mL. The overall correlation coefficient for the linear relationship in the range from 10^4 to 10^{-4} catalase units/mL was r = −0.97 ($p<0.01$) and the formula for the regression line was Log FT = 2.02 − 0.327 log catalase units/mL. Each data point in the graph represents the mean of 20 tests.

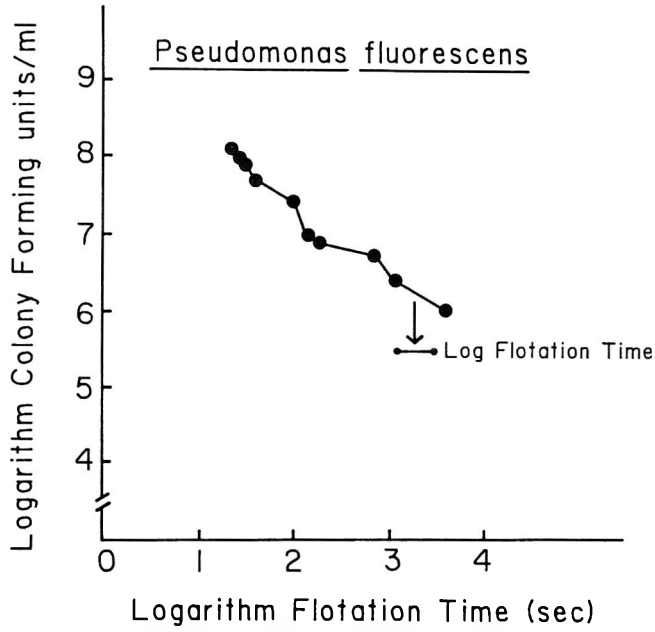

Fig. 3—Measurement of catalase activity of Pseudomonas fluorescens *by Catalasemeter with paper disc method.*

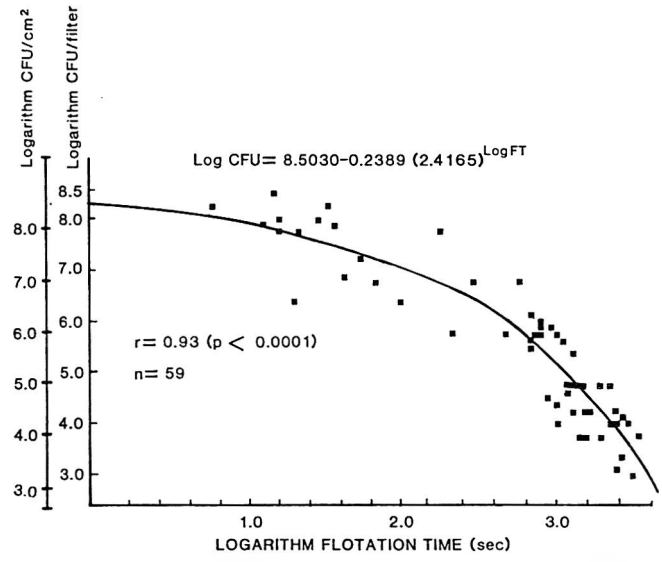

Fig. 4—Catalase activity of viable cell population from cold-stored (7°C) chicken samples in pH 3.30 phosphate buffer using Catalasemeter with membrane filter method.

Since many different bacteria contaminate food surfaces, it was necessary to obtain information on the catalase activities of some representative bacteria in pure cultures. Figures 2 and 3 show the relationship between the logarithm of colony forming unit versus the logarithm of catalase activity determined by Catalasemeter with paper disc method for *Micrococcus luteus* and *Pseudomonas fluorescens*. As expected, catalase-negative bacteria (*Lactobacillus brevis* and *Streptococcus faecalis*) showed no response in the catalase tests (data not shown). Under the experimental conditions used, the production of catalase varied with the species tested. *M. luteus* was the highest catalase producing bacteria and *P. fluorescens* and *Staphylococcus aureus* were the second highest. *Escherichia coli* and *Bacillus cereus* produced about the same, lesser amount of catalase. *Salmonella typhimurium* produced the least amount of catalase among these six catalase-positive bacteria tested (data not shown). The apparent limits of sensitivity of the Catalasemeter with paper disc system for the catalase-positive bacteria were as follows: *M. luteus* (log 4.8/mL), *P. fluores-*

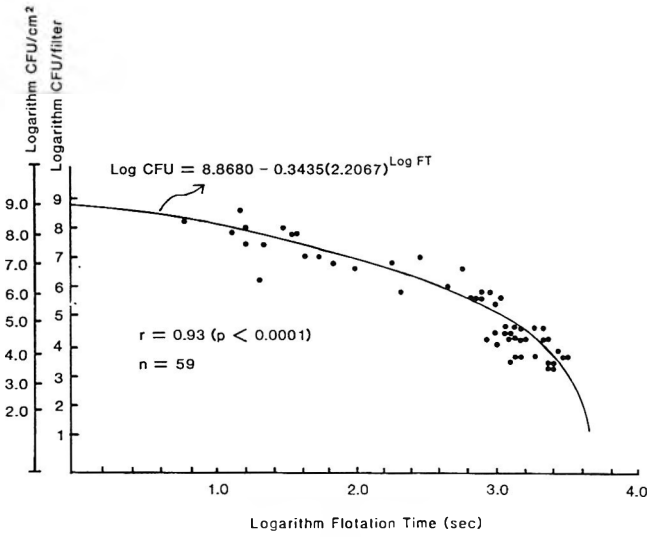

Fig. 5—Catalase activity of psychrotroph population from cold-stred (7°C) chicken samples in pH 3.30 phosphate buffer using Catalasemeter with membrane filter method.

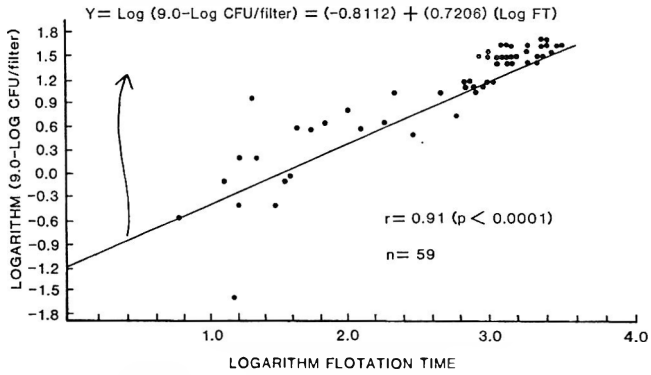

Fig. 6—Relationship between observed and predicted value of viable cell population from cold-stored chicken after linear transformation.

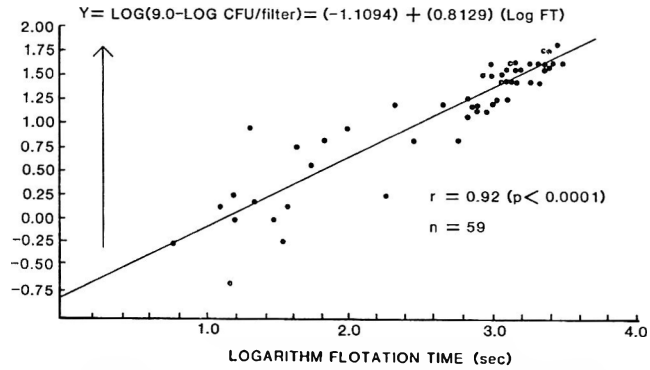

Fig. 7—Relationship between observed and predicted value of modified psychrotroph population from cold-stored chicken after linear transformation.

cens (log 6.0/mL), *S. aureus* (log 5.2/mL), *B. cereus* (log 6.2/mL), *E. coli* (log 6.8/mL), and *S. typhimurium* (log 7.9/mL).

Catalase activity of cold-stored chicken measured by catalasemeter with membrane filter system after acidified pretreatment

Figures 4 and 5 show the relationships between the logarithm of flotation time and the logarithm of CFU/cm^2 of viable cell count and modified psychrotroph count from 59 cold-stored (7°C) chicken samples, respectively. An asymptotic curve pattern was observed in both figures. The detectable limit in this methodology was about 1.6×10^3/cm^2 for a viable cell population and 4.0×10^3/cm^2 for a modified psychrotroph population. Both sets of data were used to build the standard curves for bacterial population versus flotation time. The correlation coefficient between the prediction value and observed value for both the population of viable cell and modified psychrotroph was $r = 0.93$ ($P<0.0001$).

A nonlinear model was used to analyze the relationship between log colony forming units/filter (log CFU) and log flotation time (log FT) obtained from the empirical data for the 59 chicken samples for both population of viable cells and modified psychrotrophs. The relationship between log CFU and log FT for two sets of data are shown below:

1. Viable Cell Population of 59 Chicken Samples

$$\text{Log CFU/filter} = 8.5031 - 0.2389\ (2.4165)^{\log FT}$$

2. Modified Psychrotroph Population of 59 Chicken Samples

$$\text{Log CFU/filter} = 8.8680 - 0.3435\ (2.2067)^{\log FT}$$

It was found that a linear relationship can be obtained by transforming these nonlinear equations. After this transformation, these non-linear equations were translated into linear ones as shown below:

3. Viable Cell population of 59 Chicken Samples

$$Y_1 = \text{Log}\ (9.0 - \log \text{CFU/filter}) = (-0.8112) + 0.7206\ (\log FT)$$

Modified Psychrotroph Population of 59 Chicken Samples

$$Y_2 = \text{Log}\ (9.0 - \log \text{CFU/filter}) = (-1.1094) + 0.8129\ (\log FT)$$

Based on the equations above, curves were drawn (shown in Fig. 6 and 7). These curves are all significant ($P<0.0001$). These equations can be used by the food microbiologist to estimate the microbial load on chicken samples in a comparatively short time period to enhance quality control procedures.

To utilize this information, the experimental conditions reported in this study must be followed carefully. The derived equations cannot be arbitrarily applied to other types of foods. Each type of food must be studied individually. Our conclusions in this study are based on cold-stored (7°C) chicken only.

The Catalasemeter with membrane filter system can be used to estimate the microbial load on certain food surfaces with both accuracy and rapid results. This method also has some other advantages such as flexibility, ease of performance, and saving of materials and space. The acidification necessary to differentiate blood catalase from bacteria catalase will also slightly affect bacterial catalase activity (Wang, 1985).

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Effect of pH and Salts on the Solubility of Egg White Protein

LAZAROS T. KAKALIS and JOE M. REGENSTEIN

ABSTRACT

The solubility of 1% dispersions of freeze-dried (FD) and commercial spray-dried (SD) (containing less than 0.1% SDS) egg white protein (EWP) was determined in water and in 0.1, 0.3, and 0.5M NaCl and NaI, and 0.01, 0.05, and 0.1M $Na_4P_2O_7$ at pH 3, 5, 7, and 9. Protein solubility generally increased at higher pH and salt concentrations over the ranges studied. Both I and P_2O_7 ions, but not Cl, substantially increased the apparent solubility of FDEW, but had less effect upon the solubility of SDEW. Both NaI and $Na_4P_2O_7$ changed the character of the solvent in a way similar to that of SDS, a known protein denaturant and solubilising agent. The effect of these two salts upon EWP solubility was more pronounced than for bovine serum albumin.

INTRODUCTION

THE MANNER in which a protein interacts with an aqueous solvent is a manifestation of the protein's physicochemical properties under the given conditions. A variety of protein-protein and protein-solvent interactions are involved. Many of the important functional properties of food proteins including solubility can be related to these interactions.

A thermodynamic treatment of solubility would require the establishment of an equilibrium between solid phase protein and the liquid phase. If the system contains more than one protein component, the composition of the solid phase can change upon changing the composition of the solvent and a thermodynamic treatment of solubility is not possible (Edsall and Wyman, 1958). Thus, for most purposes an operational definition is used. Kinsella (1976) summarized the current thinking about the basic steps involved in determining protein solubility as well as some of the factors that affect such measurements. One such practical method has been developed following three years of collaborative study by a USDA sponsored regional protein functionality research group (Morr et al., 1985).

Although solubility per se is important in a limited number of food applications, mostly protein-fortified beverages, it is thought to be related to other functional properties and may thus be used as an indicator of protein functionality.

Salts can affect electrostatic interactions in macromolecules by contributing to the ionic strength. However, in addition to such nonspecific effects on electrostatic interactions, salts also exhibit strikingly specific effects on the conformation of proteins which seem to have nothing to do with the sign or magnitude of the ionic charge per se (von Hippel and Schleich, 1969). The range of salt concentrations used for the elucidation of lyotropic effects is usually equal or greter than 2M (von Hippel and Schleich, 1969). However, Srinivasan and Kinsella (1982) showed that a salt concentration of 0.15M is sufficient to induce measurable changes in the water structure and the conformation of proteins.

The chemistry of egg white and its proteins has been reviewed by Vadehra and Nath (1973) and by Osuga and Feeney (1977). Cotterill et al. (1959) studied the effect of pH on the development of turbidity of one sample of fresh egg white. They found a minimum in turbidity around pH 9 which increased upon acidification of the sample. At pH 5 (where the sample was centrifuged) it decreased again. At pH 4 it rose and then decreased again at more acidic pH. Bull and Breese (1970a,b) studied the simultaneous binding of water and various solutes to ovalbumin by means of an isopiestic method.

Sodium dodecyl sulfate (SDS) is often used to improve the functionality and particularly the foaming properties of commercial egg white. Apart from reducing the surface tension, SDS also binds to egg white proteins. Hegg and Lofqvist (1977) and Shimada and Matsushita (1980) studied the delay of thermal aggregation of ovalbumin at neutral and alkaline pH in the presence of SDS, while Hegg et al. (1978) obtained similar results with conalbumin.

The present study was undertaken in order to test the applicability of the solubility measurement of Morr et al. (1985) with respect to the study of both freeze-dried and spray-dried egg white protein (EWP). The purpose of this study was to determine the effect of pH, salt concentration and type of salt (NaCl, NaI and $Na_4P_2O_7$) on solubility.

Authors Kakalis and Regenstein are with the Dept. of Poultry & Avian Sciences and Institute of Food Science, 112 Rice Hall, Cornell Univ., Ithaca, NY 14853-5601.

Table 1—Protein content of dried egg white

Method	%Protein Freeze-dried	Spray-dried
Biuret	81.34 ± 0.91	86.88 ± 0.78
Kjeldahl	81.46 ± 1.21	82.10 ± 0.69

Table 2—Percent solubility of egg white protein in water[a]

pH	Freeze-dried	Spray-dried
3	98.78 ± 0.34 a[b]	99.33 ± 0.76 a
5	96.61 ± 0.92 c	96.53 ± 0.58 c
7	97.23 ± 0.10 bc	97.46 ± 0.29 bc
9	97.64 ± 0.50 b	98.67 ± 0.62 a

[a] Determined by the Biuret method. The LSD for this set is 0.99.

[b] Treatments within a group with the same letter are not significantly different ($p>0.05$).

MATERIALS & METHODS

Samples

Eggs used for the preparation of freeze-dried egg white were from single combed White Leghorn hens, Babcock strain 200, from the Cornell University Poultry Farm. The egg white, after separation from the yolks, was given a short blend in a Waring Blendor with a flask on the surface (in order to chop the chalaza chords without air incorporation), then frozen in plastic containers, freeze-dried in a Stokes Freeze Drier (Equipment Division, Pennsalt Chemicals, Philadelphia, PA) and stored in a freezer-type plastic bag at −15°C. Spray-dried, desugared commercial egg white containing less than 0.1% added SDS (Ballas Egg Solids Div., Zanesville, OH) was kept at room temperature (~20°C), in a freezer-type plastic bag as recommended by the manufacturer.

Protein content determination

All protein determinations were based on the Biuret reaction (Gornall et al., 1949). A standard curve was produced for each run using a 10 mg/mL BSA solution (Sigma No A-4503, Lot 51F-0187) in the appropriate salt or alkaline solutions. The protein content of the BSA powder was determined spectrally, using an E1%(280-320) value of 6.6 (Sober, 1970). For the protein determination of spray-dried or freeze-dried egg white 100 mg of material was accurately weighed into a 25 mL beaker and 6 mL 1N NaOH were added. The sample was stirred with a magnetic stirrer until the protein appeared to be

Table 3—Percent solubility of egg white protein in NaCl solutions[a]

NaCl	pH 3.0	5.0	7.0	9.0
		Freeze-dried		
0.1M	94.57 ± 0.64 e[b]	95.59 ± 1.06 de	96.12 ± 1.07 de	98.46 ± 0.94 ab
0.3M	94.58 ± 0.58 e	97.22 ± 0.30 bcd	97.30 ± 0.04 bcd	99.39 ± 1.44 a
0.5M	96.57 ± 0.59 cde	97.39 ± 0.41 bcd	97.94 ± 0.81 abc	98.09 ± 1.29 ab
		Spray-dried		
0.1M	97.49 ± 0.59 g	98.42 ± 1.27 fg	98.78 ± 0.98 efg	99.39 ± 0.14 ef
0.3M	98.31 ± 0.48 fg	99.89 ± 0.33 de	99.43 ± 0.87 ef	102.62 ± 0.49 a
0.5M	100.06 ± 0.65 cde	101.21 ± 1.09 bcd	101.32 ± 0.10 abc	101.79 ± 1.31 ab

[a] Determined by the Biuret method. The LSD for the freeze-dried treatments is 1.46 and 1.35 for the spray-dried treatments.
[b] Treatments within a group with the same letter are not significantly different ($p>0.05$).

Table 4—Percent solubility of egg white protein in NaI solutions[a]

NaI	pH 3.0	5.0	7.0	9.0
		Freeze-dried		
0.1M	101.88 ± 0.03 e[b]	108.03 ± 0.56 c	109.44 ± 1.15 ab	108.55 ± 0.30 bc
0.3M	85.53 ± 0.16 f	107.56 ± 1.40 c	109.88 ± 0.42 a	109.09 ± 0.18 ab
0.5M	70.20 ± 0.42 g	105.15 ± 0.28 d	107.84 ± 0.39 c	109.12 ± 0.51 ab
		Spray-dried		
0.1M	96.43 ± 0.28 f	98.21 ± 0.38 e	99.96 ± 0.96 d	100.69 ± 0.36 bcd
0.3M	72.74 ± 0.39 g	100.57 ± 1.03 cd	100.27 ± 0.35 cd	102.00 ± 1.27 a
0.5M	67.52 ± 0.98 h	101.80 ± 0.14 ab	101.26 ± 0.38 abc	100.29 ± 0.66 cd

[a] Determined by the Biuret method. The LSD for the freeze-dried treatments is 1.05 and 1.18 for the spray-dried treatments.
[b] Treatments within a group with the same letter are not significantly different ($p>0.05$).

Table 5—Percent solubility of egg white protein in $Na_4P_2O_7$ solutions[a]

$Na_4P_2O_7$	pH 3.0	5.0	7.0	9.0
		Freeze-dried		
0.01M	104.98 ± 0.10 e[b]	108.06 ± 0.31 d	108.68 ± 0.41 cd	109.16 ± 1.31 bc
0.05M	104.59 ± 0.75 e	109.07 ± 0.14 bc	108.01 ± 0.32 d	109.62 ± 0.32 ab
0.10M	110.41 ± 0.58 e	109.22 ± 0.30 bc	108.55 ± 0.32 cd	110.38 ± 0.49 a
		Spray-dried		
0.01M	98.25 ± 0.84 ef	99.81 ± 0.35 d	99.32 ± 0.91 de	101.51 ± 0.20 bc
0.05M	98.47 ± 0.71 def	102.89 ± 0.27 ab	102.82 ± 0.50 abc	103.45 ± 0.53 a
0.10M	97.80 ± 1.07 f	101.51 ± 0.25 c	102.67 ± 0.82 abc	103.64 ± 1.82 a

[a] Determined by the Biuret method. The LSD for the freeze-dried treatments is 0.92 and 1.38 for the spray-dried treatments.
[b] Treatments within a group with the same letter are not significantly different ($p>0.05$).

completely dissolved, which required approximately 1 hr for the freeze-dried egg white and about 6 hr for the spray-dried material. The solution was transferred quantitatively to a 10 mL volumetric flask and was brought to the mark with 1N NaOH. The contents of the flask were mixed thoroughly and 0.5 mL aliquots were dispensed in three separate test tubes. After the addition of 0.5 mL water to each tube, the regular Biuret procedure was followed. The protein content of egg white was also measured by the Kjeldahl procedure (AOAC, 1965).

Protein solubility determination

Egg white, 500 mg, was accurately weighed into a 150 mL beaker. Forty milliliters of the salt solution being studied was measured into a 50 mL graduated cylinder and approximately 1 mL of that solution was added to the dry sample and stirred with a glass rod until a smooth paste was formed. A magnetic stirring bar and the rest of the 40 mL volume of the salt solution were added and the beaker was placed on a magnetic stirrer. As soon as stirring began, the pH of the solution was adjusted to the desired value with 0.1N HCl or 0.1N NaOH. Stirring continued for 1 hr at room temperature at a rate that just failed to produce a vortex. During that time, a pH electrode was retained in the dispersion and the pH was kept within 0.05 unit of the desired value. Stirring was discontinued after 1 hr, the dispersion was quantitatively transferred to a 50 mL volumetric flask and was brought to volume with the salt solution being studied. The contents of the flask were mixed uniformly and divided between two 50 mL polycarbonate centrifuge tubes that were spun at 20,000 × g (SS34 rotor, Sorvall RC2B, 16,000 rpm) for 30 min at approximately 4°C, some infranatant was removed from the centrifuge tubes by means of a Pasteur pippette and filtered. The protein content of this aliquot was determined in triplicate by the Biuret method. The solubility of two samples was also determined by the Kjeldahl procedure. Twenty milliliters of filtered infranatant were concentrated in a 500 mL Kjeldahl flask in the presence of 5 mL of concentrated H_2SO_4. The Kjeldahl procedure was followed to determine the percent protein.

The standard deviation of the Biuret measurements ranged from 0.1% to 1.4%, with almost all of the measurements having a standard deviation below 1%.

Statistical analysis

Analysis of variance was done on the solubility data. The F test was significant in all cases. A Least Significant Difference test was done to determine whether differences observed were significant or not.

RESULTS & DISCUSSION

THE PROTEIN CONTENT of the egg white used in these experiments (average of 7 determinations) are shown in Table 1. The higher value obtained with the Biuret method for the spray-dried egg white should probably be attributed to the presence of SDS. Addition of SDS up to a final concentration of 0.1% to the aqueous solution of BSA caused an increase in

the slope of the standard curve. The presence of SDS in the vicinity of the complex formed between the peptide bonds and the cupric ion changed the intensity of the absorbance. However, changes in the BSA standard curve with SDS present might not completely compensate for the changes observed with SDS and spray-dried egg white.

The solubility of both types of egg white showed a minimum at pH 5.0 and increased on both sides of this pH (Table 2). The spray-dried powder was significantly more soluble than freeze-dried protein at pH 9.0. These proteins exhibited solubility values equal to or greater than 96.6% under all conditions studied.

In the case of NaCl solutions (Table 3), a small but significant increase in solubility with higher pH values was generally observed. Results are explainable in terms of the preferential though moderate binding to proteins of chloride ions vs sodium ions (Bull and Breese, 1970a). The chloride ions bind to egg white proteins at acidic pH reducing the proteins' net positive charge and thus decreasing their solubility. As the ionic strength of the solution increases, solubility differences due to pH tend to decrease. In 0.5M NaCl solubility increases only slightly with pH. The salt concentration is high enough to mask the pH effects by providing sufficient counterions.

In NaI solutions, solubility values at 0.1M ionic strength for freeze-dried egg white exhibited a clear increase as the pH increased from 3 to 5 and then tended to level off (Table 4). A similar behavior was observed at higher ionic strength but there the decrease in solubility from pH 5 to pH 3 was much larger. In the case of spray-dried egg white, the large decrease in solubility at pH 3 and high ionic strengths was also observed; the overall solubility was slightly lower than that of freeze-dried egg white, but similar to that of spray-dried egg white in NaCl solutions.

A measurement of solubility at 0.5M salt concentration and pH 9 by means of the Kjeldahl method gave a percent solubility value of 99.79 ± 1.10 (average of three measurements). This suggests that solubilities higher than 100% by the Biuret method may be due to solvent induced spectral effects that differ from those of BSA. This is consistent with the chaotropic effect of the iodide ions (von Hippel and Schleich, 1969; Bull and Breese 1970a). The absence of a NaI-induced increase in solubility as compared to that of NaCl at alkaline pH in the presence of SDS may be due to the possibility that the SDS has already brought about the changes in protein conformation that would have been caused by NaI.

One possible explanation for the striking reduction of solubility at pH 3 as the ionic strength increases is extensive binding of iodide ions to the protein with a subsequent reduction of the net protein charge and aggregation. At pH 5, which is closer to the isoelectric points of the major egg white proteins than pH 3, aggregation did not occur. At pH 3 aggregation due to a charge decrease is less likely to take place, provided that the presence of salt caused no major shifts in the proteins' isoelectric points. Another possible explanation is a limited binding of iodide ions to the positively charged protein followed by a conformational change and aggregation. The fact that this behavior was observed only at pH 3 suggests that carboxyl groups might be involved, probably in the conformational change that follows iodide binding.

In the case of the pyrophosphate solutions (Table 5), the considerably higher than 100% solubility values of freeze-dried egg white can result from changes in the apparent solubility as perceived by the Biuret test, similar to those already mentioned in the case of NaI solutions. A measurement of solubility at 0.1M $Na_4P_2O_7$ concentration and pH 9 by means of the Kjeldahl method gave a percent solubility value of 99.59 ± 1.15. Spray-dried egg white was not as strongly affected by pyrophosphate. The presence of SDS may have caused changes such that ion effects are not as strongly perceived. Because the change of pH will have a greater effect on the ionic strength of a pyrophosphate solution than on NaI and NaCl, the possibility that ionic strength was superimposed on pH changes must be kept in mind.

A novel feature is the increased solubility of the freeze-dried sample at pH 3 for the highest $Na_4P_2O_7$ concentration used. The high buffering capacity of the pyrophosphate solution necessitated the use of considerable amounts of acid to adjust the pH to 3. The increased solubility may be due to the high ionic strength of this solution, though this effect was only observed with the freeze-dried sample. In the case of spray-dried egg white, the decreased solubility at pH 3 can be attributed to the negative effect of SDS on protein solubility at acidic pH. It is also possible that the heat-induced changes in spray-dried egg white protein were simply expressed more at this pH.

Specific ion effects

The different salts affected the solubility of egg white protein differently. Since the cation was common to the three salts studied, differences can be attributed to the anions. Moreover, it is known that monovalent cations have little effect on the conformational stability of proteins (von Hippel and Schleich, 1969). Chlorides are in the middle of the Hofmeister series and they do not have any particular effect on protein conformation. Solubility values in NaCl solutions can be regarded as a reference point for comparisons with solubility values in other salt solutions of equal ionic strength. Iodides significantly increased the solubility of freeze-dried egg white and this is in agreement with their classification as destabilizers and salting-in agents. No information was found in the literature about the position of pyrophosphates in the Hofmeister series. One would expect that large ions with small charge density would function as water structure breakers and consequently as protein structure destabilizers, primarily because of the steric stresses involved in accommodating them in the water lattice (von Hippel and Schleich, 1969). However, comparison should be made on the basis of salt concentration rather than ionic strength since classification in the Hofmeister series is based on salt concentration (von Hippel and Schleich, 1969). This is not possible with sodium pyrophosphate solutions because they become saturated at a lower concentration than the values studied here for the other salts. There is evidence that the lyotropic effect of some salts is a linear function of salt concentration up to 0.15M (Srinivasan and Kinsella, 1982). Since the observed effect for pyrophosphate ions, despite their lower concentration, was higher than that for iodide ions one may suggest that $Na_4P_2O_7$ is a more effective salting-in agent than NaI.

CONCLUSIONS

Solubility measurements using the Biuret method does not allow the investigator to consistently distinguish between real and apparent changes in egg white protein solubility. Determination of the soluble protein by means of the Kjeldahl method can be very helpful in this respect, as sample measurements have shown. Spectroscopic color methods should be used with caution since a change of solvent may affect the absorbance of certain chromophores. Both NaI and $Na_4P_2O_7$ appeared to change the character of the solvent in a way similar to that of SDS, a known protein denaturant and solubilising agent, and thus should be classified as salting-in compounds.

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Antigenicity of Ovomucoid Remaining in Boiled Shell Eggs

JAINXIN GU, TSUKASA MATSUDA, and RYO NAKAMURA

ABSTRACT

Antigenicity of egg white proteins which remained in heated shell eggs was analyzed quantitatively by using rabbit antibodies specific to egg white proteins and human antibody from patients allergic to egg. The major antigenic component in heated (100°C for 20 min) and coagulated egg white was ovomucoid. A considerable amount of ovomucoid remained immunoreactive even after heating at 100°C for 15 min, though ovomucoid in stored eggs was a little less stable to heating than that of fresh eggs. Even long-time heating (100°C for 45 min) could not completely eliminate the ovomucoid immunoreactivity to human IgE antibody.

INTRODUCTION

CHICKEN EGG WHITE is a food or ingredient with both high nutritional value and good functional properties. On the other hand, chicken egg is well known to be a typical food allergen (Breneman, 1978). Some people, especially babies and infants, are hypersensitive to egg white, and such hypersensitive reactions have been reported to be caused by several protein antigens in egg white (Taylor, 1980).

Ovomucoid is a major allergenic protein in chicken egg white (Langeland, 1982a, 1982b; Hoffman, 1983), and is well known as a highly heat-stable protein with trypsin inhibitory activity (Osuga and Feeney, 1977). Bleumink and Young (1969) reported that ovomucoid prepared from boiled eggs still caused a skin reaction in allergic patients. Many reports showed that ovomucoid was physically stable to heating (Stevens and Feeney, 1963), and that heat-induced conformational changes of ovomucoid were highly reversible (Matsuda et al., 1981a). Like some other allergenic proteins, ovomucoid allergenicity was associated with such heat stability of ovomucoid antigenic activity (Taylor, 1980; Matsuda et al. 1982). Thus, there are many studies on the ovomucoid heat stability using isolated ovomucoid, whereas the effect of heating in the presence of other egg white proteins on the ovomucoid antigenic activity is not still well known.

The aim of the present study was to obtain basic information on the reduction of allergenic activity of egg white proteins by heat processing of shell eggs.

MATERIALS & METHODS

Chicken egg

Fresh eggs of White Leghorn hens were obtained from the Laboratory of Animal Nutrition, School of Agriculture, Nagoya University. The fresh eggs were collected on the first, the 8th, the 10th and the 12th days, and stored at 4°C for 12, 4, 2, and zero days, respectively. These eggs (0–12 days of storage) were heat-treated at the same time on the 12th day, and used for the preparation of soluble protein fractions described below.

Authors Gu, Matsuda, and Nakamura are with the Dept. of Food Science & Technology, School of Agriculture, Nagoya Univ. Chikusa-ku, Nagoya 464, Japan.

Heat treatment and preparation of soluble proteins

The shell eggs were put into a water bath (25°C) and the temperature of the bath was raised from 25°C to 90° or 100°C at a rate of about 7°C/min. After being kept for 5–45 min in the bath maintained at the desired temperature, the eggs were cooled in an ice water bath for 30 min. A piece of coagulated egg white of heat-treated shell eggs was cut apart and homogenized with equal amount (by weight) of phosphate buffered saline, pH 7.4 (PBS) in a Potter-Elvehjen type homogenizer. The suspensions of homogenized egg white were centrifuged at 1500 × *g* for 20 min to remove aggregated proteins. The supernatant solutions were used as soluble protein fractions for the following experiments. Raw egg white was treated in the same manner as the coagulated egg white, and used as a soluble protein fraction of unheated eggs.

Electrophoresis

The sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis was performed according to Laemmli (1970). The gel slab was stained with 0.2% Coomassie Brilliant Blue R-250, and destained with 7% acetic acid containing 10% methanol.

Antisera

The antiserum to egg white proteins was prepared by immunizing a rabbit with homogenized raw egg white. The egg white (50 μL) was mixed with 950 μL 20 mM phosphate buffer, pH 7.4, containing 0.15M NaCl, emulsified with 1 mL of Freund's complete adjuvant, and injected subcutaneously three times at 2-wk intervals. Bleeding was performed 10 days after the last injection, the serum was separated and stored at −80°C. The antiserum to ovomucoid was prepared as described previously (Matsuda et al., 1982). The human sera of allergic patients were obtained from the Nagoya University Hospital, and the sera with high titer of IgE antibody to ovomucoid were selected as described previously (Matsuda et al., 1985).

Immunoelectrophoresis and immunoelectrodiffusion

Immunoelectrophoresis (Grabar and Williams, 1953) was performed in gel plates of 1% agarose in barbital buffer (pH 8.6, I = 0.025). About 6 μL of a 1:50 dilution of the soluble fractions of heated or raw egg white was added to each well, and electrophoresis was carried out at 5 V/cm for 60 min. The anti-egg white serum (100 μL of a 1:2 dilution) was added to each trough, and allowed to diffuse for 16 hr. The gel plates were washed with PBS for 3 days, and stained with 5% Amido Black 10B in methanol containing 10% acetic acid, and destained with 2% acetic acid.

Immunoelectrodiffusion (rocket immunoelectrophoresis) was done according to Laurell (1966) in gel plates of 1.2% agarose in the barbital buffer containing 2% (by vol.) antiserum to ovomucoid. The antigen solutions (6 μL) diluted as above were loaded in each well and electrophoresed at 5 V/cm for 16 hr. The gel plates were washed, stained and destained as above.

Enzyme-linked immunosorbent assay (ELISA)

The antigenic activity of ovomucoid in heated eggs was investigated also by the competitive inhibition analysis of ELISA (Engvall and Perlmann, 1971) using human IgE antibody. Flat-bottomed microtiter plates were coated with an ovomucoid solution (0.1 μ/mL), and 100 μL of competitors (serially diluted soluble fractions of egg white with or without heating) were mixed with 50 μL antibody solution (1:300 dilution of the human serum), and added to the wells. The human IgE antibody which reacted with the plate-bound ovomucoid were determined by using a peroxidase-coupled anti-human IgE antibody (Cappel Labs. Inc.) as described previously (Matsuda et al, 1983).

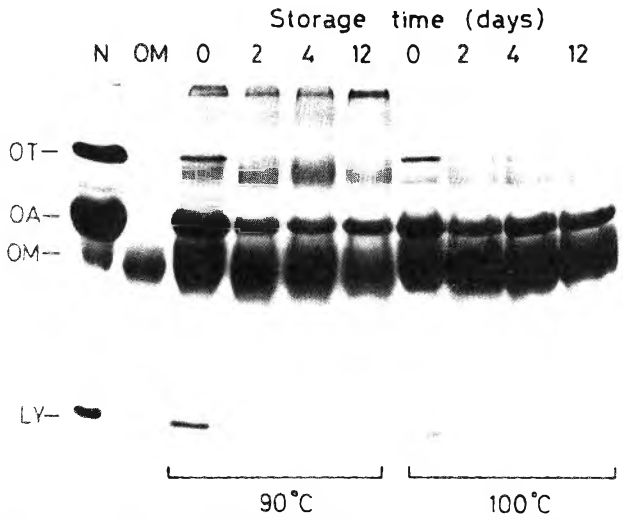

Fig. 1—SDS polyacrylamide gel electrophoresis of soluble fractions prepared from egg white of heated shell eggs. Shell eggs were heated at 90°C or 100°C for 20 min after storage for 0, 2, 4 and 12 days. Unheated egg white (N) and purified ovomucoid also were applied for comparison (the dilution of unheated egg white was about ten times that of the soluble fractions). OT, OA, OM and LY indicate the bands of ovotransferrin, ovalbumin, ovomucoid and lysozyme, respectively.

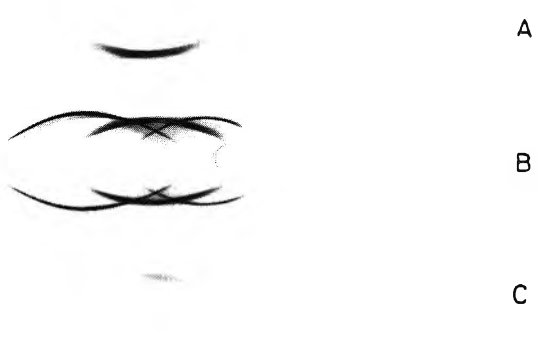

Fig. 2—Immunoelectrophoresis of soluble fraction prepared from egg white of heated shell eggs. Eggs were heated at 100°C for 20 min after 12 days of storage (C) or without storage (A). The raw egg white diluted similarly was applied for comparison (B).

RESULTS

THE PROTEINS in the egg-white soluble fraction were analysed by SDS-gel electrophoresis (Fig. 1). The most abundant protein of the soluble fractions was ovomucoid. Lysozyme was found in the soluble fractions of fresh eggs, whereas little or no lysozyme could be found in that of stored eggs. The amount of both ovalbumin and ovotransferrin which remained soluble in heated fresh-eggs was larger than those in heated stored-eggs.

The immunoreactivity of proteins which remained soluble after heating in fresh and stored eggs was analysed by immunoelectrophoresis using the anti-egg white serum. Clear precipitin arcs of major egg white proteins (ovalbumin, ovotransferrin, ovomucoid and lysozyme) were formed against raw egg white, whereas the precipitin arc of these proteins, except ovomucoid, could not be detected for the soluble fractions of heated eggs (100°C for 20 min) (Fig. 2). Only a strong arc of ovomucoid and another weak arc (not identified) were formed against heated eggs, and the arc intensity of ovomucoid was stronger for the fresh egg than for the stored egg (12 days).

The effect of storage time on the heat stability of ovomucoid

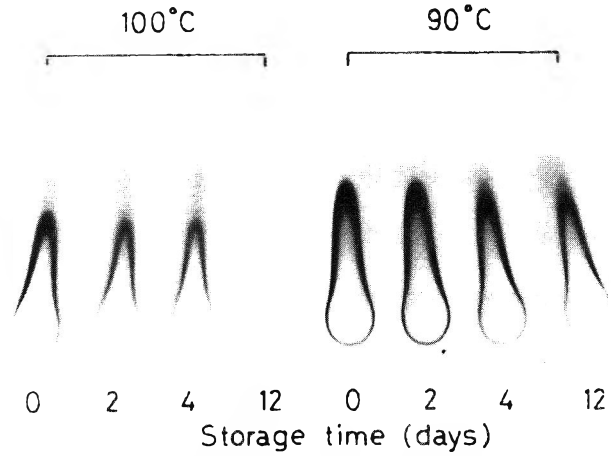

Fig. 3—Immunoelectrodiffusion of soluble fractions prepared from eggs heated after storage for various periods. Eggs were heated at 90° or 100°C for 20 min after storage for 0, 2, 4 and 12 days.

antigenic activity was investigated by a quantitative immunodiffusion method. Ovomucoid remained immunologically active in heated eggs (2–12 days of storage) (Fig. 3). The antigen in stored eggs, especially in the 12 day-stored egg, formed a precipitin rocket shorter and fainter than that of the antigen in fresh eggs.

Immunoelectrodiffusion of the soluble fraction prepared from fresh and stored (12 days) eggs after heating at 100°C for various periods is shown in Fig. 4. The sample from fresh egg formed a precipitin rocket even after being heated for 45 min, though the rocket was a little less in height and intensity than that of the sample prepared from raw egg white. On the other hand, the stored egg (12 days) produced almost no precipitin rocket after heating for 35 min or more.

The binding of ovomucoid from unheated and heated eggs to human IgE was investigated by a sophisticated method of ELISA competitive inhibition analysis (Fig. 5). The concentration of the soluble fraction from heated eggs required to attain 50% inhibition of the reaction between ovomucoid and human IgE was about 10 times greater than that of raw eggs. The immunoreactivity of ovomucoid in stored eggs appeared a little more sensitive to heating than that of fresh eggs. However, a few percent of ovomucoid in egg white remained immunoreactive even after heating at 100°C for 45 min.

DISCUSSION

A CONSIDERABLE AMOUNT of ovomucoid remained soluble in heat-induced coagulated eggs with or without storage, which is in agreement with the results previously reported on the aggregation of egg white proteins heated below 90°C (Matsuda et al., 1981b). Ovomucoid is highly resistant to heat-induced aggregation and coagulation even in boiled shell eggs. However, the soluble ovomucoid appeared to be denatured partly by heating especially in stored eggs because of its reduced immunoreactivity.

The antigenicity of ovomucoid in the stored egg (12 days) was reduced by heating more strongly than that of fresh eggs. Also, the antigenicity of ovomucoid in stored eggs without heating was slightly less than that of fresh eggs. The pH of egg white increased with storage time; the pH was 7.6, 9.0 and 9.2 for fresh, 4 day- and 12 day-stored eggs, respectively. The increase in pH of egg white and/or some unknown changes in other components in egg white during storage might reduce the antigenicity of ovomucoid and the heat stability of ovomucoid.

The effect of heating time on the heat-induced inactivation of the antigenicity of ovomucoid indicates that the antigenic

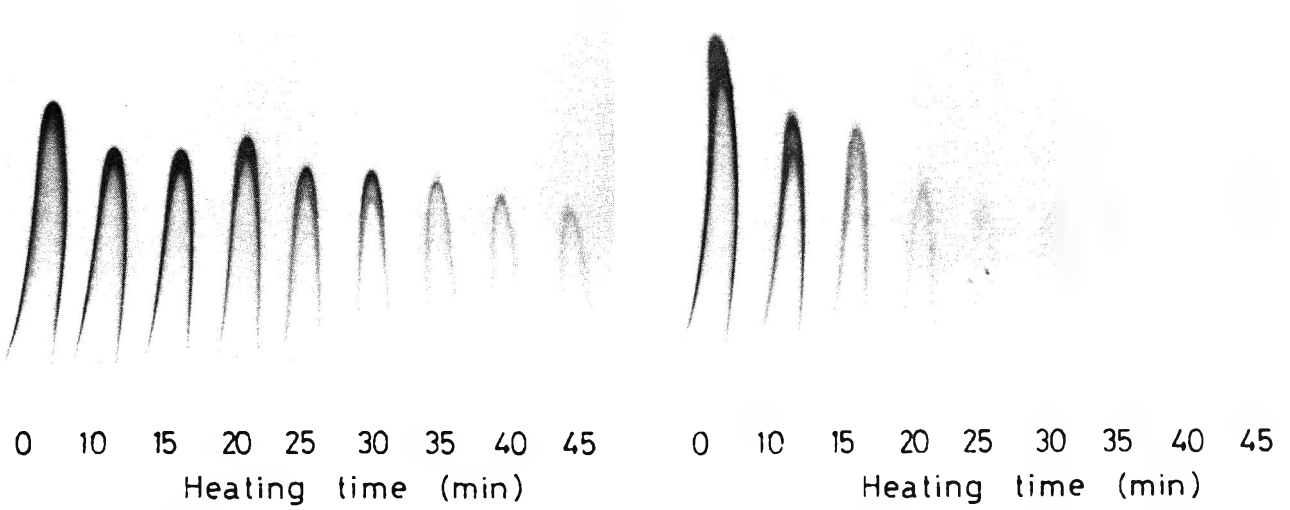

Fig. 4—Immunoelectrodiffusion of soluble fractions prepared from eggs heated for various periods. Eggs were heated at 100°C for 0–45 min after 12 days of storage (right) or without storage (left).

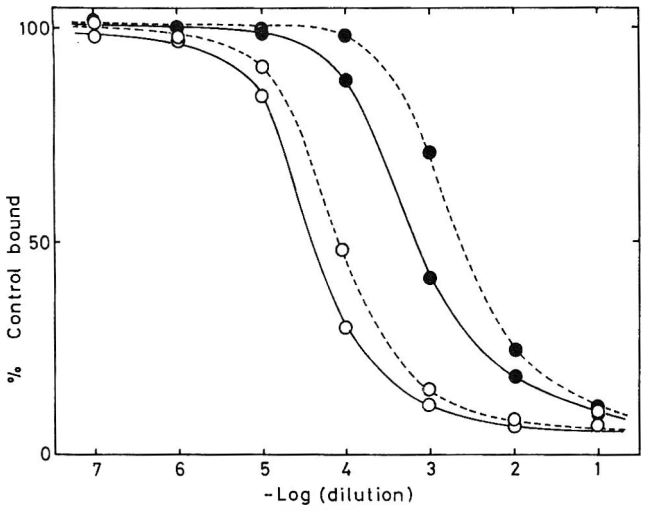

Fig. 5—ELISA competitive inhibition analysis for ovomucoid antigenic activity in heated shell eggs. Eggs were heated at 100°C for 45 min (●) after 12 days of storage (-----) or without storage (——), and the soluble fractions prepared from the egg whites were diluted serially (1:10-1:1,000,000). The unheated samples (○) also were analysed for comparison. Each point represents the mean of two determinations.

activity of ovomucoid in shell eggs, especially in fresh eggs cannot effectively be reduced by the heat treatment (100°C for 15 min) usually used for boiled eggs. Heating at 100°C for 30 min or more seems to be required to attain the effective loss of the antigenic activity of ovomucoid in shell eggs. A considerable amount of antigenic ovomucoid was detected in the fresh egg heated at 100°C for 30 min, but the heat stability of the antigenic activity of ovomucoid in fresh eggs would not be very important in practice, because fresh eggs stored for one day or less are not commonly consumed.

The binding of ovomucoid to human IgE antibody did not completely disappear after heating for 45 min, which appeared to be a little inconsistent with the results using the rabbit antiovomucoid antibody that no immunoprecipitation rocket was detected for the stored egg heated for 45 min. This might be due to the difference in reaction specificity between rabbit IgG and human IgE antibodies; the binding site of human IgE might be distinct from that of rabbit IgG. It is possible that the antigenicity of ovomucoid molecule could be destroyed by heating but the allergenicity of IgE binding could be retained. Also, in the present study, the antigenicity was examined only for protein antigens in the soluble fractions extracted from coagulated egg white. Some aggregated or coagulated proteins might retain their immunoreactivity. Thus, egg allergic individuals could not safely consume even eggs boiled for a long period of time.

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Changes in Microbiological, Chemical, Rheological and Sensory Characteristics during Ripening of Vacuum Packaged Manchego Cheese

M. NUÑEZ, P. GAYA, M. MEDINA, M. A. RODRÍGUEZ-MARÍN, and C. GARCÍA-ASER

ABSTRACT

The effects of cheese age when packaged and of packaging material permeability on Manchego cheese characteristics were studied throughout a 6-month ripening period. Cheese age when packaged had no significant effect on microbial levels, proteolysis, lipolysis or flavor. However, moisture content and rheological characteristics were significantly different for cheeses packaged 1, 2 or 3 wk after salting. Packaging material had no influence on microbiological, chemical, rheological or sensory characteristics of Manchego cheese. Packaged cheese had a lower flavor intensity than nonpackaged cheese, but no significant differences in flavor quality between packaged and nonpackaged cheese were recorded. Weight losses after 6 months were undetectable in packaged cheese, but they averaged 15.16% in nonpackaged cheese.

INTRODUCTION

MANCHEGO CHEESE is the main variety of cheese manufactured in Spain, with a production over 30,000 tons/year. Seasonal variations in milk production and market fluctuations are responsible for differences in age and lack of homogeneity in Manchego cheese characteristics when marketed. Cheese may be stored for up to 10 months, with profuse external mold development, heavy mite attach to the rind and high weight losses. Repeated brushing and coating with olive oil protects rind from mold growth and mite damage, but economic losses due to water evaporation are not suppressed.

Since Stine (1950) patented a procedure for rindless block Swiss cheese, vacuum packaging of long term storage cheese with multilayer barrier films has been satisfactorily applied to varieties such as Cheddar, Edam, Emmenthal, Gouda, Grana Padano, Kachkaval, Saint Nectaire, etc. (Didienne et al., 1978; Fradin, 1980; Kojev, 1982; Massoni et al. 1985). Reduction of weight losses due to water evaporation and excessive rind formation, prevention of mold development and mite attack, unnecessary control of relative humidity in ripening rooms and labor reduction during cheese storage are the main advantages claimed for this curing procedure (Fradin, 1984). However, defects due to excessive gas production by starter bacteria or by contaminating heterofermentative lactobacilli (Hettinga et al., 1974; Keller and Jaarsma, 1975) may arise in packaged cheese. Cheese held at 15°C may develop a considerable pressure when packaged in gas-tight films (Scott, 1981). Consequently, lower curing temperatures and materials of a higher CO_2 permeability are recommended to avoid texture defects in cheese with eyeholes.

The objective of the present work was to investigate ripening of vacuum packaged Manchego cheese in order to determine the conditions (cheese age when packaged, packaging material, etc.) suitable to obtain 6-month old vacuum packaged Manchego cheese with physicochemical and sensory characteristics as close as possible to those of Manchego cheese ripened without packaging for 1 month at 10°C.

The authors are affiliated with the Departamento de Bioquímica y Microbiología, Instituto Nacional de Investigaciones Agrarias, Apartado 8111, Madrid 28040, Spain.

MATERIALS & METHODS

Cheesemaking

Four 500L vats of Manchego cheese were manufactured on different days from pasteurized (75°C/15 sec) milk, inoculated with 0.005% frozen concentrated starter cultures. After 30 min, rennet and 0.01% $CaCl_2$ were added; coagulation took place in 35 min at 30°C. Curds were cut into 5 mm cubes and cooked for 15 min at 38°C. Cheeses, 22 cm in diameter and 9 cm high, were pressed overnight, salted for 48 hr and ripened for 6 months.

The effects of cheese age when packaged on Manchego cheese characteristics were studied on cheeses from vats 1 and 2 (duplicate experiment), manufactured with a *Streptococcus cremoris* + *S. lactis* starter (Miles-Martin S.A.E., Spain). Three lots of cheese (seven cheeses/lot) from each vat were vacuum packaged after 1 (lot P1), 2 (lot P2), or 3 (lot P3) wk storage at 12°C/70% RH in bags of BK-1 film (Grace S.A., Spain). Bags were sealed with metal clips and shrunk by heat (90°C), and the cheeses held at 4°C for 6 months from manufacture. Two additional lots from each vat were ripened without packaging at 4°C/90% RH (lot N1) or at 10°C/87% RH (lot N2) for the same period.

The effects of packaging material permeability were studied on cheeses from vats 3 and 4 (duplicate experiment), manufactured with a *S. lactis* + *S. cremoris* + *Leuconostoc cremoris* starter (Amerex, Spain). Three lots of cheese (seven cheeses/lot) from each vat were packaged after 1 wk storage at 12°C/70% RH into bags of BK-1 (lot P4), BK-3 (lot P5) or BK-5 (lot P6) films (Grace S.A., Spain) as described above, and held at 4°C for 6 months from manufacture. Medium O_2 permeabilities were 200, 300, and 375 mL/24 hr·m^2·bar for BK-1, BK-3 and BK-5 films, respectively, whereas minimum CO_2 permeabilities increased from 500 to 1400 mL/24 hr·m^2·bar, according the manufacturer's specifications. Two additional lots from each vat were ripened without packaging at 4°C/90% RH (lot N3) or at 10°C/87% RH (lot N4).

Sampling

A different cheese from each lot was selected at random for analysis when the lot was packaged (on day 10 for lots P1, P4, P5 and P6; on day 17 for lot P2; on day 24 for lot P3) and after 2, 4 and 6 months from manufacture. A reference (nonpackaged cheese ripened for 1 month at 10°C/87% RH) Manchego cheese from each vat was selected at random for analysis. Representative samples from the interior and the rind (5 mm deep) of cheese were obtained according to Law et al. (1973) and Nuñez (1978), respectively.

Microbiological analyses

Cheese homogenates and decimal dilutions were prepared as described by Nuñez et al. (1985). Total counts and coliforms were determined after Clark et al. (1978) and Hartman et al. (1978), respectively. Staphylococci were estimated on plates of egg yolk tellurite glycine pyruvate agar incubated at 37°C for 48 hr, suspicious colonies being tested for coagulase production (Baird-Parker, 1979). Yeasts and molds were enumerated on plates of potato dextrose agar (pH 3.5) after incubation at 24°C for 96 hr.

Chemical determinations

Moisture content and pH were determined according to AOAC (1980) and Medina et al. (1982), respectively. Nonprotein nitrogen (NPN),

Table 1—Microbiological and chemical characteristics[a] of 6-month old Manchego cheese packaged[b] 1, 2 or 3 wk after salting and of 6-month old nonpackaged[c] Manchego cheese

Characteristic	Interior					Rind				
	P1	P2	P3	N1	N2	P1	P2	P3	N1	N2
Log total counts/g	7.14	7.17	7.70	7.23	6.76	6.55	6.57	6.20	6.78	7.14
Log coliforms/g	0.70	0.99	0.45	2.28	0.65	0.30	0.00	0.00	0.15	0.00
Log staphylococci/g	1.96	1.61	2.10	1.71	3.16	3.85	4.48	2.30	4.00	4.26
Log yeasts/g	2.36	2.07	3.57	2.18	1.74	3.27	3.80	4.64	5.75	6.31
Log molds/g	0.85	0.74	2.22	0.00	1.91	1.92	1.85	2.73	3.34	5.02
Moisture (%)	38.47	37.32	37.28	35.95	35.38	35.66	34.21	32.74	17.33	16.46
pH	5.36	5.38	5.41	5.33	5.41	5.45	5.49	5.47	5.51	5.49
NPN[d]	17.18	16.69	18.56	17.10	19.64	ND[f]	ND[f]	ND[f]	ND[f]	ND[f]
FFA[e]	11.43	10.82	11.33	10.68	10.32	ND[f]	ND[f]	ND[f]	ND[f]	ND[f]

[a] Data on the table are mean values from two cheesemaking trials.
[b] P1, P2, P3: cheeses packaged under BK-1 film 1, 2 or 3 wk after salting and ripened at 4°C/90% RH.
[c] N1, N2: nonpackaged cheeses ripened at 4°C/90% RH or at 10°C/87% RH.
[d] NPN level is expressed as the percentage of total nitrogen.
[e] FFA content is expressed as mequiv/100 g fat.
[f] ND: not determined.

Table 2—Levels of significance[a] of main effects (sample location[b], treatment[c] and cheese age at the time of analysis[d]) on characteristics of Manchego cheese packaged 1, 2 or 3 wk after salting and of nonpackaged Manchego cheese

Characteristic	Level of significance			Characteristic	Level of significance	
	Sample	Treatment	Age		Treatment	Age
Total counts	**	NS	***	NPN	NS	**
Coliforms	***	NS	***	FFA	NS	*
Staphylococci	***	NS	NS	Eap	***	***
Yeasts	***	*	***	Breaking force	***	***
Molds	***	***	***	Hardness	***	***
Moisture	***	***	NS	Flavor quality	NS	NS
pH	***	NS	*	Flavor intensity	***	NS

[a] Levels of significance: *P<0.05; **P<0.01; ***P<0.001; NS: nonsignificant.
[b] Sample location: interior or rind of the cheese.
[c] Treatment: cheese packaged 1, 2 or 3 wk after salting and nonpackaged cheese ripened at 4° or 10°C.
[d] Age: 2, 4 or 6 months of ripening.

obtained after precipitation of a cheese homogenate with 12% (final concentration) trichloroacetic acid and filtration through Whatman No. 42 paper, and total nitrogen (TN) were determined in duplicate by the Kjeldahl method. NPN was expressed as the percentage of TN. Free fatty acids (FFA) were determined in duplicate after extraction of cheese fat and titration with 0.1N ethanolic KOH solution as described by Nuñez et al. (1986). FFA content was expressed as meq/100g fat.

Rheological determinations

Six cylinder-shaped samples (17 mm height × 17 mm diameter) from each cheese were compressed to 80% of their original height after 4 hr at room temperature (20–22°C), using an Instron Compression Tester 1122 (Instron Ltd., England) with crosshead and chart speeds of 50 and 500 mm/min, respectively. Apparent elastic modulus (Eap) was calculated from the slope of the initial part of the compression curves. Force at breaking point (breaking force) and hardness, defined as the work done on the cheese after 80% compression (Law and Wigmore, 1982), were measured from the compression curves.

Sensory evaluation

Samples (15-mm cubes), coded with three-digit random numbers, were presented in plastic disposable dishes after holding cheese overnight at room temperature (20–22°C). Order of sample presentation to each panelist was randomized. Bread and water were used as rinsing agents between samples.

Cheese flavor quality was assessed by a trained 10-member tasting panel on a hedonic scale from 1 (dislike extremely) to 9 (like extremely). Cheese flavor intensity was assessed by the same panel on a scale from 1 (much milder than reference) to 5 (much stronger than reference) with a 3-point score corresponding to flavor intensity of 1-month old reference Manchego cheese given to panelists as an external standard. Cheese firmness (panel), defined as the force required to bite through the sample (Culioli and Sherman, 1976), was judged by panelists on a scale from 1 (extremely soft) to 9 (extremely firm). A 5-point score corresponded to firmness of 1-month old reference Manchego cheese, given to panelists as an external standard.

One-month old reference cheeses for sensory evaluation of 2, 4 and 6-month old experimental cheeses were selected at random from cheeses made at the same dairy and under the same conditions as experimental cheeses, 1, 3, or 5 months later, respectively. They were ripened without packaging at 10°C/87% RH.

Statistical treatment of data

After a log transformation for microbial counts, analyses of variance were performed on data obtained at different stages of ripening, with sample location, treatment and cheese age at the time of analysis as main effects, using program BMD08V (Dept. Biomathematics, UCLA, Los Angeles). Correlation coefficients (r) of linear regressions were calculated by means of program BMD01R (Dept. Biomathematics, UCLA, Los Angeles).

RESULTS & DISCUSSION

Characteristics of 1-month old reference Manchego cheese

Levels (log cfu/g) of total counts, coliforms, staphylococci, yeasts and molds in the interior of 1-month old reference Manchego cheese ripened without packaging at 10°C/87% RH (mean values for vats 1, 2, 3 and 4) were 8.65, 4.73, 3.49, 3.16, and 1.99, respectively, whereas these levels at the rind were 8.43, 2.94, 4.38, 5.68, and 4.70, respectively. Moisture content averaged 41.73% in the interior and 25.54% at the rind, while the respective pH values were 5.12 and 5.50. Mean NPN level was 8.94% and mean FFA content 9.49 meq/100g fat. Average values for rheological characteristics calculated from compression curves were 0.33 N/mm^2 as Eap, 25.57N as breaking force and 0.31j as hardness.

Influence of age when packaged on Manchego cheese characteristics

Microbiological and chemical characteristics of 6-month old Manchego cheese packaged 1, 2 or 3 wk after salting held at 4°C and of nonpackaged cheese held at 4° or 10°C are summarized in Table 1. Mean levels (log cfu/g) of total counts, coliforms, staphylococci, yeasts and molds in the interior of 10-day old cheese were 8.48, 5.06, 2.45, 4.16, and 1.00, respectively. All microbial groups decreased gradually in the interior of both packaged and nonpackaged cheese throughout ripening. No coagulase positive staphylococci were detected

Table 3—Rheological and sensory characteristics[a] of 6-month old Manchego cheese packaged[b] 1, 2 or 3 wk after salting and of 6-month old nonpackaged[c] Manchego cheese

Characteristic	P1	P2	P3	N1	N2
Eap (N/mm^2)	0.34	0.57	0.80	1.38	1.22
Breaking force (N)	24.66	31.00	35.21	45.53	39.82
Hardness (j)	0.34	0.42	0.47	0.53	0.52
Flavor quality[d]	6.35	6.65	6.60	6.80	6.75
Flavor intensity[e]	3.25	3.25	3.50	3.60	3.70

[a] Data on the table are mean values from two cheesemaking trials.
[b] P1, P2, P3: cheeses packaged under BK-1 film 1, 2 or 3 wk after salting and ripened at 4°C/90% RH.
[c] N1, N2: nonpackaged cheeses ripened at 4°C/90% RH or at 10°C/87% RH.
[d] Flavor quality was determined on a 1 (dislike extremely) to 9 (like extremely) points hedonic scale.
[e] Flavor intensity was determined on a 1 (much milder than reference) to 5 (much stronger than reference) points scale, a 3-point score corresponding to 1-month old reference Manchego cheese, ripened without packaging at 10°C/87% RH, given to panelists as an external standard.

Table 4—Microbiological and chemical characteristics[a] of 6-month old Manchego cheese packaged[b] in BK-1, BK-3 or BK-5 films and of 6-month old nonpackaged[c] Manchego cheese.

Characteristic	Interior					Rind				
	P4	P5	P6	N3	N4	P4	P5	P6	N3	N4
Log total counts/g	8.13	8.25	8.23	8.48	7.85	8.13	8.07	8.26	8.39	8.18
Log coliforms/g	2.51	2.64	2.18	2.15	1.75	1.64	2.33	1.64	1.12	0.00
Log staphylococci/g	3.78	3.73	3.84	3.47	3.87	3.58	1.36	1.41	2.16	0.50
Log yeasts/g	0.74	1.56	1.54	1.02	0.00	4.27	4.27	4.31	5.10	5.02
Log molds/g	0.50	0.00	1.24	0.89	2.70	2.80	2.32	2.45	4.88	5.37
Moisture (%)	42.05	41.35	42.08	35.14	35.02	41.64	41.36	40.66	19.20	18.41
pH	5.22	5.08	5.12	5.06	5.08	5.22	5.14	5.19	5.17	5.17
NPN[d]	12.14	15.06	12.77	12.14	13.56	ND[f]	ND[f]	ND[f]	ND[f]	ND[f]
FFA[e]	9.04	11.19	10.64	10.05	10.94	ND[f]	ND[f]	ND[f]	ND[f]	ND[f]

[a] Data on the table are mean values from two cheesemaking trials.
[b] P4, P5, P6: cheeses packaged under BK-1, BK-3 or BK-5 films 1 wk after salting and ripened at 4°C.
[c] N3, N4: nonpackaged cheeses ripened at 4°C/90% RH or at 10°C/87% RH.
[d] NPN level is expressed as the percentage of total nitrogen.
[e] FFA content is expressed as meq/100g fat.
[f] ND: not determined.

after 3 wk from manufacture. Mean levels of total counts, coliforms and staphylococci at the rind of 10-day old cheese were 7.88, 2.02, and 4.48, respectively. These groups also decreased during ripening at the rind of both packaged and nonpackaged cheese. Yeasts and molds, with 5.21 and 3.79 as respective mean log counts at the rind of 10-day old cheese, decreased in packaged cheese, remained unaltered in nonpackaged cheese held at 4°C and increased considerably in nonpackaged cheese held at 10° C.

Sample location (interior or rind) affected significantly ($P<0.001$) levels of all microbial groups (Table 2). When only packaged cheese was considered, the same highly significant effect of sample location was observed for all microbial groups. Total counts and coliforms reached higher levels in the interior at the end of the ripening period, whereas aerobic salt-tolerant microorganisms such as staphylococci, yeasts and molds grew better at the rind. Visible mold development could be perceived on the surface of nonpackaged cheese from the second month of ripening. Yeasts and molds were the only microbial groups influenced by treatment (Table 2), with higher levels in nonpackaged cheese, their growth being enhanced by aerobic conditions. No significant ($P<0.05$) differences in yeast or mold counts between cheeses packaged 1, 2 or 3 wk after salting were detected. Cheese age at the time of analysis had a highly ($P<0.001$) significant effect on total counts and coliforms, with decreasing levels during ripening, and on yeasts and molds, groups with a pattern of behavior influenced by packaging, as previously pointed out.

Mean moisture content of 10-day old Manchego cheese was 44.05% in the interior and 29.31% at the rind. Mean moisture content in the interior of packaged cheese (lots P1, P2 and P3) was 39.36%, 38.62% and 37.69% after 2, 4 or 6 months, respectively. At the rind mean content was 28.28%, 33.07% and 34.20% after 2, 4 or 6 months. Interior and rind moisture contents tended to balance during ripening in packaged cheese, whereas in nonpackaged cheese the difference between moisture contents in the interior and at the rind did not vary significantly with time of aging. Mean moisture content in the interior of nonpackaged cheese (lots N1 and N2) was 39.08%, 37.60% and 35.66% after 2, 4 or 6 months. At the rind the respective contents were 19.63%, 17.92% and 16.89%.

Moisture contents in the interior of packaged and nonpackaged 6-month old Manchego cheese (Table 1) resembled data reported for 14-month old Grana Padano by Massoni et al. (1985), but moisture content at the rind of nonpackaged Manchego cheese was much lower than at the rind of nonpackaged Grana Padano. Sample location and treatment influenced significantly ($P<0.001$) moisture content (Table 2). However, cheese age at the time of analysis had no significant effect on moisture content, a highly ($P<0.001$) significant interaction age × treatment being recorded. All main effects (sample location, age when packaged and age at the time of analysis) were stastically significant ($P<0.01$) when only packaged cheese was considered.

The pH increased in the interior of cheese from a mean value of 5.04 for 10-day old cheese to values over 5.30 for 6-month old cheese, whereas pH at the rind averaged 5.56 in 10-day old cheese and remained unchanged throughout maturation. Treatment had no significant influence on pH (Table 2), but sample location had a highly ($P<0.001$) significant effect on it.

NPN increased from a mean level of 5.41% in 10-day old cheese to values reported in Table 1 for 6-month old cheeses, with no significant differences between packaged and nonpackaged cheeses or between cheeses packaged 1, 2 or 3 wk after salting. Data reported for Grana Padano by Massoni et al. (1985) do not show either a significant effect of treatment on NPN levels. FFA content increased slightly throughout maturation, from an average value of 8.23 meq/100g fat in 10-day old cheese to values over 10 meq/100g fat after 6 months, with no significant effect of treatment on lipolysis (Table 2). Higher counts of yeasts and molds at the rind of nonpackaged cheese did not enhance proteolysis nor lipolysis.

Considerable differences between the rheological characteristics of packaged and nonpackaged cheese were observed (Table 3). Treatment and cheese age at the time of analysis had

Table 5—Levels of significance[a] of main effects (sample location[b], treatment[c] and cheese age at the time of analysis[d]) on characteristics of Manchego cheese packaged in BK-1, BK-3 or BK-5 film and of nonpackaged Manchego cheese

Characteristic	Level of significance			Characteristic	Level of significance	
	Sample	Treatment	Age		Treatment	Age
Total counts	*	NS	***	NPN	NS	***
Coliforms	NS	***	***	FFA	NS	NS
Staphylococci	**	NS	***	Eap	***	***
Yeasts	***	NS	NS	Breaking force	***	***
Molds	***	***	*	Hardness	***	***
Moisture	***	***	***	Flavor quality	NS	NS
pH	***	*	NS	Flavor intensity	***	NS

[a] Levels of significance: *P<0.05; **P<0.01; ***P<0.001; NS: nonsignificant.
[b] Sample location: interior or rind of the cheese.
[c] Treatment: cheese packaged in BK-1, BK-3 or BK-5 film and nonpackaged cheese ripened at 4° or 10°C.
[d] Age: 2, 4 or 6 months of ripening.

Table 6—Rheological and sensory characteristics[a] of 6-month old Manchego cheese packaged[b] in BK-1, BK-3 or BK-5 films and of 6 month-old non-packaged[c] Manchego cheese

Characteristic	P4	P5	P6	N3	N4
Eap (N/mm²)	0.32	0.32	0.37	1.19	1.41
Breaking force (N)	20.98	20.45	22.51	45.92	47.08
Hardness (j)	0.29	0.29	0.32	0.59	0.61
Flavor quality[d]	6.20	6.55	6.30	6.60	6.80
Flavor intensity[e]	3.05	3.45	3.30	4.80	4.60

[a] Data on the table are mean values from two cheesemaking trials.
[b] P4, P5, P6: cheeses packaged in BK-1, BK-3 or BK-5 film 1 wk after salting and ripened at 4°C.
[c] N3, N4: nonpackaged cheeses ripened at 4°C/90% RH or at 10°C/87% RH.
[d] Flavor quality was determined on a 1 (dislike extremely) to 9 (like extremely) points hedonic scale.
[e] Flavor intensity was determined on a 1 (much milder than reference) to 5 (much stronger than reference) points scale, a 3-points score corresponding to one-month old reference Manchego cheese, ripened without packaging at 10°C/87% RH, given to panelists as an external standard.

highly (P<0.001) significant effects (Table 2). Creamer and Olson (1982) reported significant correlations between moisture content and breaking force for Cheddar cheese of low (r=0.849) and medium (r=0.811) pH. In our study, all three rheological characteristics determined were significantly correlated to moisture content, with r values exceeding 0.90 in all cases. Proteolysis was negatively correlated with hardness in Cheddar cheese (Fedrick and Dulley, 1984), but the same correlation was not statistically significant (r=0.183) for Manchego cheese. When only packaged cheese was considered, cheese age when packaged influenced significantly (P<0.01) Eap, breaking force and hardness.

Lee et al. (1978) observed significant correlations between hardness (panel) and breaking force (r=0.701) or force after 80% compression (r=0.946), whereas Chen et al. (1979) reported a significant correlation (r=0.845) between hardness (panel) and hardness (Instron). In the present work, significant correlations (P<0.01) between firmness of 6-month old Manchego cheese as judged by panelists and rheological characteristics determined from compression curves were obtained, with r=0.882 for Eap, r=0.924 for breaking force and r=0.913 for hardness.

Flavor quality was not influenced by treatment (Table 2), and no significant differences between cheeses packaged 1, 2 or 3 wk after salting were detected either. However, a significant (P<0.001) effect of treatment on flavor intensity was recorded (Table 2), packaged cheeses receiving the lowest scores (Table 3). Cheese packaged 1 or 2 wk after salting exhibited the mildest flavor, close to that of 1-month old reference Manchego cheese (flavor intensity = 3), but differences between cheeses packaged 1, 2 or 3 wk after salting were not statistically significant.

It may be concluded from data in Table 1 that cheese age when packaged had no significant influence on microbiological or chemical characteristics of packaged Manchego cheese, except moisture content. However, data in Table 3 show that Manchego cheese packaged 1 wk after salting and held at 4°C for 6 months was the most similar in rheological and sensory characteristics to 1-month old reference Manchego cheese ripened, nonpackaged, at 10°C. Storage at a lower temperature, i.e. 2°C, may retard even more proteolysis and lipolysis according to Nuñez et al. (1986), minimizing differences in chemical characteristics between packaged and reference cheese.

Influence of packaging material on Manchego cheese characteristics

All microbial groups decreased during ripening in the interior of both packaged and nonpackaged cheese. At the rind, mold numbers increased after six months, but only in nonpackaged cheese (Table 4). Mean levels (log cfu/g) of total counts, coliforms, staphylococci, yeasts and molds were 9.05, 6.55, 5.04, 2.74 and 1.60, respectively, in the interior of 10-day old cheese, and 8.88, 5.35, 5.35, 5.60 and 3.75, respectively, at the rind.

Yeasts and molds were the microbial groups more affected (P<0.001) by sample location, with higher levels at the rind. Cheese age at the time of analysis had a highly (P<0.001) significant effect on total counts, coliforms and staphylococci (Table 5). Coliforms and molds were the only microorganisms influenced by treatment, with higher levels of coliforms in packaged cheese and higher mold counts in nonpackaged cheese. No significant effect of packaging material on any microbial group was detected.

Mean moisture content of 10-day old Manchego cheese was 43.71% in the interior and 33.94% at the rind. Interior and rind moistures reached an equilibrium after six months in packaged cheese, with differences under 2% in all cases (Table 4). No significant effect of wrapping material on moisture content was detected when only packaged cheese was considered. Differences in moisture content between interior and rind of nonpackaged cheese exceeded 15% after six months. Weight losses at that time were undetectable in packaged cheese, whereas they averaged 14.66% and 15.66% in nonpackaged cheese held at 4°C and 10°C, respectively. These losses are considerably higher than those reported for Grana Padano after 12 months of storage (8.7–12.3%) by Massoni et al. (1985).

Values of pH increased in the interior from 5.07 in 10-day old cheese to levels in Table 4, whereas a slight decrease was observed at the rind from a mean pH value of 5.31 recorded for 10-day old cheese.

In 10-day old cheese mean level of NPN was 7.02% and

mean FFA content 9.09 meq/100g fat. The lower proteolysis detected after 6 months in cheese from vats 3 and 4 (Table 4) when compared to cheese from vats 1 and 2 (Table 1) may be due to a weaker proteolytic activity of the *S. lactis* + *S. cremoris* + *L. cremoris* starter. Values of pH were lower for cheese from vats 3 and 4, a highly significant correlation ($r = 0.856$) between pH values and NPN levels being recorded. No significant differences in proteolysis or lipolysis between packaged and nonpackaged cheese were detected (Tables 4 and 5). Proteolysis and lipolysis were not affected either by packaging material.

Highly significant ($P<0.001$) effects of treatment and cheese age on rheological characteristics were detected (Tables 5 and 6). Eap, breaking force and hardness were significantly correlated to moisture content, with r values exceeding 0.98 in all cases. Packaging material had no significant effect on the rheological characteristics of Manchego cheese. Production of CO_2 by starter bacteria or by contaminating microorganisms was not high enough to cause formation of large eyeholes, split defects or differences in texture detectable from compression curves between cheeses packaged under the various materials.

Packaging of cheese did not affect its flavor quality (Table 5), but flavor intensity scores were significantly ($P<0.001$) higher for nonpackaged cheese. No significant differences in flavor quality or intensity due to packaging material were observed.

Data in Tables 4 and 6 confirm the validity of conclusions drawn from the first experiment (Tables 1 and 3). Six-month old Manchego cheese with characteristics close to those of 1-month old reference cheese ripened without packaging at 10°C may be obtained packaging the cheese 1 wk after salting and holding it at 4°C, or at a lower temperature. The procedure may be used to overcome seasonal shortage in Manchego cheese production, improving curing homogeneity throughout the year.

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Effect of Fluorescent Light on the Isomerization of Retinyl Palmitate in Skim Milk

A. M. GAYLORD, J. J. WARTHESEN, and D. E. SMITH

ABSTRACT

Skim milk fortified with retinyl palmitate was exposed to fluorescent light and analyzed for the isomers of retinyl palmitate. High performance liquid chromatography was used to separate and identify the all-*trans* and some *cis* forms. Besides all-*trans* retinyl palmitate, small amounts of 13-*cis* retinyl palmitate were present in unexposed skim milk. The level of all-*trans* decreased over the 48-hr period of light exposure. The 13-*cis* levels also decreased but not as rapidly. The level of the 9-*cis* isomer increased over the 48-hr period of light exposure.

INTRODUCTION

THE INCREASE in use of transparent containers for packaging milk and the increased consumption of lowfat milk has raised concern over the light stability of vitamin A in milk. Rates of vitamin A loss in milk have been studied and the initial mechanism of light-induced destruction of vitamin A is considered to be photoisomerization (Senyk and Shipe, 1981; deMan, 1981). The extent to which this photoisomerization of the all-*trans* form occurs in milk exposed to light has not yet been investigated. By understanding the reaction mechanism and predicting rates of loss, possible ways of preventing vitamin A loss in milk can be determined.

High performance liquid chromatography (HPLC) methods for the determination of vitamin A isomers in food products have been reported (Egberg et al., 1977; Thompson et al., 1980; Stancher and Zonta, 1982a, b). Advantages of HPLC methods over conventional methods of vitamin A analysis are that HPLC methods allow for the separation of the *cis*-isomers of vitamin A which have lower biological potencies than the all-*trans* form (Ames, 1966).

The purpose of this study was to separate, identify and quantify retinyl palmitate isomers present in skim milk as a function of fluorescent light exposure in order to obtain a better understanding of the mechanism of light-induced destruction of vitamin A in milk.

MATERIALS AND METHODS

HPLC SEPARATION of retinyl palmitate isomers was accomplished using a 10μm particle size, 25 cm Hibar II, Lichrosorb silica column (Merck, Inc.). One percent ether in hexane was used as an eluant at a flow rate of 1.5 mL/min. A Waters model M-6000A HPLC pump was used with a model 440 absorbance detector containing a 313 nm filter for co-chromatographic identification and for quantification of the isomers. A Kratos Model 757 Spectroflow variable wavelength HPLC detector was used to provide information on the absorbance maxima of the isomers.

Qualitative analyses of the retinyl palmitate isomers present in exposed and unexposed skim milk samples were performed using standards of 9-*cis*, 11-*cis*, and 13-*cis* retinyl palmitate (obtained from Hoffman La Roche, Inc.). The all-*trans*-retinyl palmitate was obtained from Sigma Chemical Co. UV absorbance maxima for each retinyl palmitate isomer present in the milk extract was determined by making repeated HPLC runs at different detector wavelengths and measuring peak height. Detector settings at intervals of every two nm were used for the range 313–340 nm.

Commercially available fortified skim milk packaged in two-quart paperboard containers (Pure Pak) was used as the source of milk because of the decreased likelihood of any prior exposure to light. Glass test tubes (13 × 100 mm) which had a capacity of 8 mL were completely filled with skim milk and closed with Teflon-lined screw caps. The filled tubes were laid horizontally and held in place by a rack while exposed to fluorescent light at an intensity of 150 ft-c (1614 lux) at 4°C in light chambers mounted with fluorescent lights (General Electric Cool White No. F15T8-CW). A rheostat was used to adjust the light intensity, as measured by a Type 214 General Electric light meter. Five replicate samples were removed from the light at 0, 6, 12, 24, and 48 hr of exposure.

Retinyl palmitate was extracted from the milk samples using the hexane extraction method of Thompson et al. (1980) with the following modification. The extracts were concentrated threefold by removing 3 mL of the hexane layer, evaporating the solvent with a stream of nitrogen and resuspending with 1 mL of hexane. Recovery of added all-*trans* retinyl palmitate averaged 94.9%. Since reliable quantitative *cis* standards were not available, peak areas of each isomer were integrated by a Hewlett Packard 3390A integrator and the areas were compared to the area of an external standard of all-*trans* retinyl palmitate. Precautions were taken to minimize the exposure of samples and standards to light during handling. Analyses of variance (Steel and Torrie, 1980) were performed to determine the significant differences between mean levels of the isomers found in the skim milk samples for each exposure time.

RESULTS & DISCUSSION

USING THE CHROMATOGRAPHIC conditions described above, baseline separation of 9-*cis*, 13-*cis* and all-*trans* retinyl palmitate was achieved (Fig. 1). The 11-*cis* and 13-*cis* isomer standards were not resolved at the baseline but because the 11-*cis* isomer was not present in either the unexposed or exposed skim milk samples, this did not interfere with either the qualitative or quantitative isomer determinations.

The all-*trans* retinyl palmitate standard contained approximately 5% of the 13-*cis* isomer. The all-*trans* standard solution (Fig. 1) and the unexposed skim milk extract (Fig. 2) contained approximately the same ratio of 13-*cis* to all-*trans*-retinyl palmitate (1:20). Thompson et al. (1980) also demonstrated the presence of small amounts of the 13-*cis* isomer along with the all-*trans* isomer in fortified milk.

A peak eluting between the 13-*cis* and all-*trans* isomer (retention time approximately 10 min) was observed in skim milk which had been exposed to fluorescent light (Fig. 2). Evidence for the identification of the peak eluting between the 13-*cis* and all-trans isomers was achieved using co-chromatography with known standards. When the skim milk hexane extract was spiked with the 9-*cis* standard, an increase in the height of the second peak was observed. The 13-*cis* and all-*trans* retinyl palmitate were also identified by this method. The 11-*cis* isomer appeared not to be present using this method of identification.

Rando and Chang (1983) studied iodine catalyzed isomerization reactions of all-*trans* retinyl palmitate and found that very little of the 11-*cis* isomer was generated. It appears that the light-induced isomerization of retinyl palmitate in milk is similar in that 11-*cis* was not detected.

The authors are with the Dept. of Food Science & Nutrition, Univ. of Minnesota, 1334 Eckles Ave., St. Paul, MN 55108.

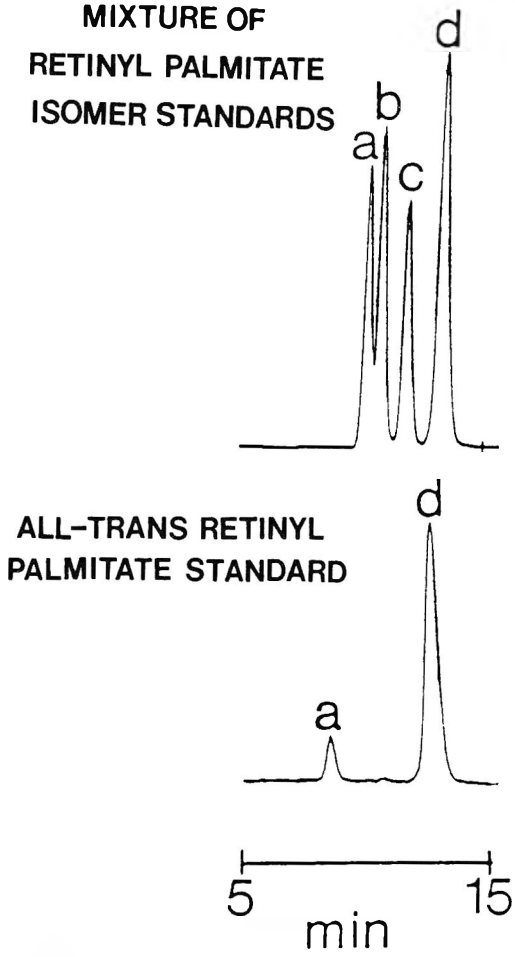

*Fig. 1—Chromatograms of retinyl palmitate standards: (a) 13-*cis*; (b) 11-*cis*; (c) 9-*cis*; and (d) all-*trans.

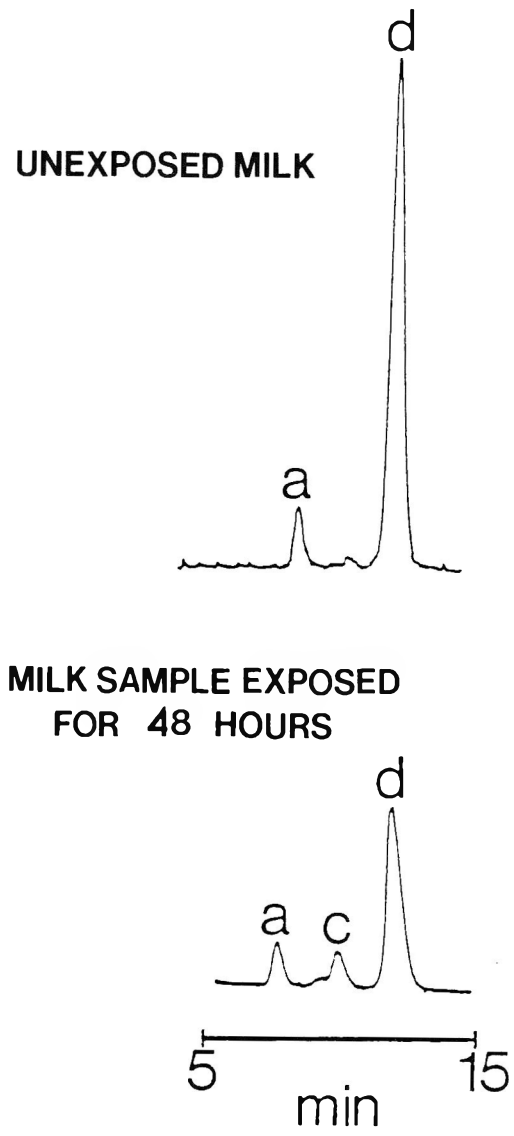

*Fig. 2—Chromatograms of retinyl palmitate isomers in skim milk: (a) 13-*cis*; (c) 9-*cis*; and (d) all-*trans.

Further qualitative analysis was done by determining the UV absorbance maxima for each peak (Table 1). The experimental values obtained are very close to the expected values determined by Stancher and Zonta (1982a). The literature values given were determined in hexane-dioxane, whereas the experimental results were determined in hexane-ether. Therefore, differences in solvent polarity could explain the spectral shifts observed. Another explanation for the slight differences could be the specificity of the variable wavelength absorbance detector used in the HPLC system. The close agreement of the maximum absorbance values provide further evidence for the positive identification of the 13-*cis* and 9-*cis* isomers present in the skim milk samples.

Quantification of the retinyl palmitate *cis* isomers found in skim milk is difficult due to the unavailability of good quantitative standards and the low levels of *cis* isomers that are present in milk. Initially, very little of the 9-*cis* isomer was present and the mean value reported in Table 2 for the control sample is approaching the limits of detection under the chromatographic conditions used for analysis.

The concentration of the 13-*cis* isomer of retinyl palmitate decreased slowly over the 48-hr exposure period (Table 2). The changes in concentration are not significantly different until 24 hr of light exposure. The 24 hr and 48 hr analyses show the same mean values for the amount of 13-*cis* retinyl palmitate present. Based on the results of Rando and Chang (1983), the 9,13-di-*cis* isomer could coelute with the 13-*cis* isomer, so these values may include both the 13-*cis* and the 9,13-di-*cis* isomer. Rando and Chang found that the 13-*cis* isomer isomerized further to both 9-*cis* and 9,13 di-*cis*-retinyl palmitate, which could explain this apparent plateau. The levels of the 13-*cis* isomer would likely be affected by the rate of formation from all-*trans* as well as the rate of degradation induced by continued light exposure.

*Table 1—Absorbance maxima for 9-*cis*, 13-*cis*, and all-*trans *retinyl palmitate*

Isomer	Absorbance maxima (nm) Experimental	Literature[a]
9-*cis*	324	322
13-*cis*	330	328
all-*trans*	326	326

[a] from Stancher and Zonta (1982a), determined in hexane-dioxane.

*Table 2—Concentration of 13-*cis*, 9-*cis*, and all-*trans *retinyl palmitate in skim milk exposed to a light intensity of 150 ft-c (1614 lux) at 4°C*

Hours	Concentration of retinyl palmitate isomers (μg/mL) 13-*cis*	9-*cis*	all-*trans*
0	$0.11^{a} \pm 0.01$	$0.01^{a} \pm 0.004$	$1.31^{a} \pm 0.03$
6	$0.11^{a} \pm 0.01$	$0.02^{ab} \pm 0.005$	$1.06^{b} \pm 0.05$
12	$0.10^{ab} \pm 0.02$	$0.04^{bc} \pm 0.01$	$0.97^{c} \pm 0.08$
24	$0.08^{b} \pm 0.01$	$0.07^{cd} \pm 0.02$	$0.71^{d} \pm 0.02$
48	$0.08^{b} \pm 0.01$	$0.09^{d} \pm 0.02$	$0.50^{e} \pm 0.03$

[a-e] Each value is the mean of five replicates ± standard deviation. Values with the same superscript letter are not significantly different within each column.

The formation of the 9-*cis* isomer upon exposure of the all-*trans* form to light was observed in hexane by Mousseron-Canet (1971). Low levels of the 9-*cis* isomer in cheese were observed by Stancher and Zonta (1982b). However, the formation of this isomer in milk or other food systems as a function of light exposure has not been previously reported.

The presence of the 9-*cis* isomer appears to be a qualitative indicator of retinyl palmitate degradation. The level of the 9-*cis* isomer shows a steady increase over the 48 hr of exposure. The amount of 9-*cis* retinyl palmitate formed during exposure does not correspond on an equimolar basis to the loss of all-*trans* or all-*trans* and 13-*cis* loss. The amount of 9-*cis* formed accounts for about 10% of the original all-*trans* retinyl palmitate lost after 48 hr. Since the amount of the 9-*cis* isomer formed does not correspond to the amount of all-*trans* retinyl palmitate lost, it is likely that other degradation products not picked up by this chromatographic procedure are formed.

Within 48 hr of exposure to fluorescent light, approximately 70% of the all-*trans* retinyl palmitate was lost. The calculated rate constant based on a first order degradative mechanism is $0.0198\ hr^{-1}$.

The 9-*cis*, 13-*cis*- and all-*trans* isomers of retinyl palmitate were separated, identified and quantified in skim milk after various times of light exposure. The 9-*cis* isomer can serve as a useful qualitative indicator of light-induced degradation of all-*trans* retinyl palmitate. Separation and quantification of the retinyl palmitate isomers is useful in predicting actual biological activity of the vitamin A present in milk. Using conventional methods of vitamin A analysis, the actual biological activity of vitamin A in fortified foods is over estimated if *cis*-isomers are present. The HPLC method employed has advantages over other methods of vitamin A analysis. It allows for separation of different isomers of retinyl palmitate which differ in biological potencies so that a more accurate estimation of biological activity can be obtained.

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Influence of Heat Treatment of Milk on the Flow Properties of Yoghurt

ESTELLE M. PARNELL-CLUNIES, Y. KAKUDA and J.M. DEMAN

ABSTRACT

Milk was processed by vat heating (VAT, 85°C for 10 to 40 min), high temperature-short time heating (HTST, 98°C for 0.5 to 1.87 min) and ultra-high-temperature heating (UHT, 140°C for 2 to 8 sec) and made into yoghurt. Apparent viscosity (shear rate 57.75^{s-1}) ranged from 550–568 mPa.sec for VAT treatments, 260–462 mPa.sec for HTST treatments and 170–333 mPa.s for UHT treatments. Flow behavior indices for all treatments were between 0.55 to 0.65 while consistency index values ranged from 0.3 to 1. Milk processed by HTST systems appeared to be viable alternatives to vat processing. UHT processed milk, however, yielded yoghurts of low viscosity.

INTRODUCTION

YOGHURT and other cultured milk products have traditionally been significant contributors to the diets of European and Middle Eastern populations. Increased consumption of yoghurt in North America has occurred mostly in the last 20 years and is probably related to the introduction of fruit and flavored yoghurts (Kalab et al., 1983).

A main criterion of quality assessment of yoghurt is the physical properties of the gel. Physical characteristics depend upon the type of yoghurt, with 'set style' yoghurt exhibiting a firm gel while 'stirred' yoghurt has a semi-liquid consistency. The latter is produced by mechanical agitation of the coagulum prior to packing in retail containers.

Physical properties of yoghurt are influenced by milk composition and manufacturing conditions. Variables affecting physical properties include heat treatment applied to milk, protein content, homogenization, acidity, culture, mechanical handling of coagulum and the presence of stabilizers (Nielson, 1975; Rasic and Kurmann, 1978).

Heating of milk to temperatures exceeding normal pasteurization temperatures (72°C, 16 sec) is known to improve the consistency of yoghurt. There is, however, considerable controversy regarding temperature-time relationships required to yield a product with optimum stability. The trend towards continuous milk processing systems has presented problems in converting process lines from batch to continuous while maintaining an equivalent product. Heat treatments of 85°C for 30 min or 90–95°C for 5–10 min were cited by Tamine and Deeth (1980), but an earlier survey compiled by Rasic and Kurmann (1978) mentioned heat treatments encompassing a range from 75–150°C with residence times from 2 sec to 30 min depending on temperature.

Such divergent treatments may arise due to the following reasons: (1) varying milk composition, (2) nonstandardized experimental conditions, and (3) single point viscosity measurements. Flow behavior of yoghurt, similar to many foods, may be described as non-Newtonian. Non-Newtonian materials show a dependence of apparent viscosity on shear rate; therefore, single point viscosity measurements are often invalid for comparison since shear rates are rarely the same between studies. In addition to making comparison difficult, single point measurements offer no information on flow behavior parameters which are useful in the design of unit processes such as pumping and heat exchanging (Hermansson, 1975).

The objectives of this study were to provide and compare information on flow behavior properties of yoghurt produced from milk heated by batch (VAT) and two continuous heating systems.

The authors are with the Dept. of Food Science, Univ. of Guelph, Guelph, Ontario, Canada, N1G 2W1.

MATERIALS & METHODS

Processing

Raw, whole milk was obtained from the University of Guelph research farm or a local dairy. Milk was heated by one of three heating systems represented by the following temperature-residence time combinations: vat heating at 85°C for 10, 20, 30, and 40 min (VAT), high temperature-short time heating at 98°C for 0.5, 0.95, 1.42 and 1.87 min (HTST) and ultra-high-temperature heating at 140°C for 2, 4, 6, and 8 sec (UHT). An additional treatment utilized unheated milk as a control, for a total of 13 experimental treatments.

VAT treatments were processed in a steam jacketed batch pasteurizer to 85°C for appropriate holding times and homogenized at 50°C at 105 kg/cm^2 prior to inoculation with culture. HTST and UHT treatments were processed in a modified Cherry Burrel tubular, indirect heating system fitted with a Taylor automatic, proportional temperature controller. Different residence times were obtained by using holding tubes of varying lengths and changing pump speed. All residence times were calculated under an operating homogenization pressure of 105 kg/cm^2.

After homogenization and cooling, milk was inoculated with 3% mixed culture (*Streptococcus thermophilus* and *lactobacillus bulgaricus* 1:1 in ratio) and filled into 700 ml containers. Inoculated milk was incubated between 42° and 44°C until pH 4.6 ± 0.05 was attained (Radiometer combination glass electrode), then stored at 5°C. Each treatment was replicated three times. All processing treatments were completed over a 2-month period. Protein content of milk during this time ranged from 3.06–3.17%. Analysis of Covariance procedures (Freund and Littel, 1981) were applied to account for variation in protein content.

Viscosity measurement

All viscosity measurements were performed on 1 day old yoghurt. For relative comparison between treatments, yoghurt was subjected to a standard stirring procedure prior to viscosity measurements. Yoghurt was inverted from 700 mL containers into a Hobart Model K5A mixer with a paddle stirrer and mixed for exactly 2 min at speed setting 1.5.

Apparent viscosity was determined with a Haake Rotovisco (Model RV3, MK 500 head) fitted with a MV II sensor system. All readings were taken at 10°C maintained by a tempering vessel attached to a Haake refrigerated, circulating waterbath. Flow curves were obtained by taking readings at shear rates up to 115 sec^{-1}. Prior to commencing the experiment, calibration was performed by Haake Buchler Instruments (Saddle Brook, New Jersey) to determine the spring constant which was used to calculate appropriate factors. Shear stress (A), shear rate (M); and instrument factors (G) used were 3.423 Pa/scale

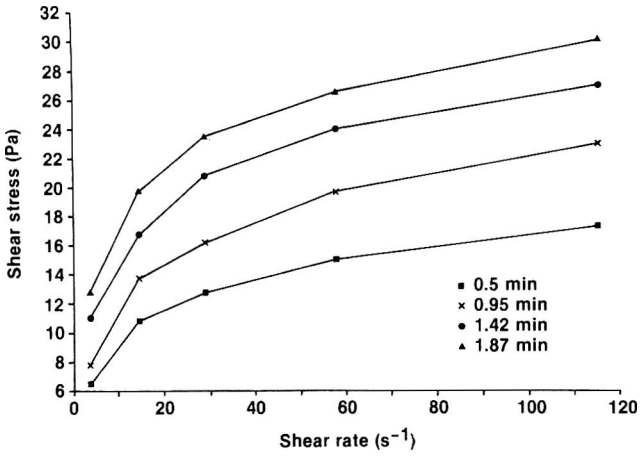

Fig. 1—Flow curves for yoghurt prepared from HTST heated milk at 98°C for 0.5 to 1.87 min.

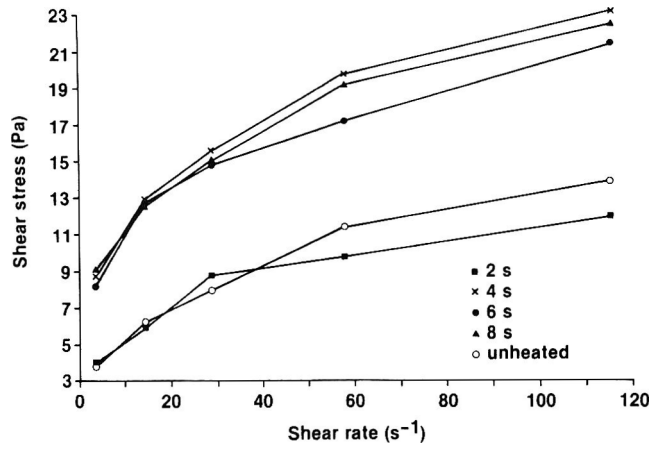

Fig. 2—Flow curves for yoghurt prepared from unheated milk and UHT heated milk at 140°C for 2 to 8 sec.

grad., 0.9 min/sec and 3,799 mPa.sec/scal grad..min, respectively. Shear stress, shear rate, and apparent viscosity were calculated as follows:

$$\text{Shear stress } (\sigma) = \text{A.S Pa}$$

$$\text{Shear rate } (D) = \text{M.n sec}^{-1}$$

$$\text{Apparent viscosity } (\eta) = \frac{\text{G.S.}}{\text{n}} \text{mPa.sec}$$

where S = scale reading; n = rpm setting.

Scale reading (S) was recorded at 15 sec after shearing commenced. Time dependent curves were recorded at constant shear of 57.7 sec^{-1} over a 10-min period. Instrument readings were relayed to an Apple compatible computer connected via an analog-to-digital converter.

Calculations

The flow properties of many foods can be described by the power law equation:

$$\sigma = KD^n$$

where σ = shear stress, D = shear rate, and K and n are power law constants. K is the consistency index and n is the flow behavior index. For Newtonian fluids, n = 1 and K is the viscosity coefficient. Another model which is applicable to some foods is the Hershel-Bulkley equation which contains a yield stress (σ_o) term:

$$\sigma - \sigma_o = KD^n$$

The value of the yield stress can be evaluated by fitting the data to the Casson equation:

$$\sqrt{\sigma} = \sqrt{\sigma_o} + K\sqrt{D}$$

Power law constants and yield stress were obtained using double logarithmic and square root plots, respectively. Data at a shear rate of 3.6 sec^{-1} were not used in calculating constants due to low instrument precision. Regression analysis was performed on data for estimation of slopes and intercepts.

RESULTS & DISCUSSION

Influence of heat treatment on flow curves

Shear stress-shear rate curves for all treatments are shown in Fig. 1 to 3. Pseudoplastic behavior was exhibited by all treatments with the existence of a yield stress. Highest shear stress readings were exhibited by VAT treatments followed by HTST and UHT treatments, respectively, and this trend was generally independent of rate of shear.

The influence of residence time was most noticeable in HTST treatments were shear stress increased from about 17 Pa to 30 Pa for times of 0.5 and 1.87 min, respectively, at the highest shear rate (Fig. 1). For UHT treatments, the 2 sec residence time resulted in substantially lower shear stress than the three longer residence times, and was very similar to the unheated control (Fig. 2). Correlation coefficients for mean (n = 3) shear stress (at shear rate 57.7sec^{-1}) versus residence time were 0.72 for the UHT system and 0.99 for the HTST system. Profiles for all yoghurt made from VAT heated milk were almost superimposable (Fig. 3).

Apparent viscosity at a mid-range shear rate was calculated to allow comparison of heat treatment effects (Table 1). Results suggest that there was a definite heat-induced change occurring in milk which affected its functionality in yoghurt manufacture. The usual explanation for improvement in consistency of yoghurt as a function of heat treatment of milk is whey protein denaturation (Grigorov, 1966; Rasic and Kurmann, 1978). This theory was disputed by Labropoulos et al. (1981a) who found a variable relationship between firmness of yoghurt and degree of whey protein denaturation in heated milk. The same trend of vat heated milk giving higher viscosity than UHT milk as reported in the present study was also found by Labropoulos et al. (1981b). For comparative purposes, degree of whey protein denaturation was determined on a subset of heated milk samples (15 samples including VAT, HTST and UHT treatments) used in this study. Degree of denaturation was determined by measuring residual native protein nitrogen in raw and heated milk samples fractionated by Rowlands (1938) procedure. Results are shown as a scatter plot in Fig. 4. Linear regression of the data showed that 80% of variation in apparent viscosity could be explained by degree of denaturation. This relatively high R^2 value should be interpreted with caution, however, since the control sample gave an apparent viscosity of 198 mPa.sec although this milk was not heat treated above 55°C (homogenization temperature) and consequently should exhibit minimal denaturation. Yoghurt from the UHT-2 sec treatment had an apparent viscosity of 170.4 mPa.sec which corresponded to a mean denaturation value of 52%. Thus, degree of denaturation appears to have little influence on apparent viscosity until the whey proteins are at least half denatured.

It is obvious from the results that the cumulative effect of temperature and residence time was substantially different among the three types of heating systems. This is especially apparent in HTST treatments where apparent viscosity was markedly influenced by residence time (r = 0.99). While results suggest a denaturation related mechanism, the range of apparent viscosities within treatments (170–570 mPa.sec) could imply that more than one physicochemical transition was occurring. An in-depth study of heat induced changes in the protein fraction of heated milk is currently in progress and results will be the subject of a future publication.

Influence of heat treatment on rheological constants

Typical Casson and log-log plots for HTST treatments are shown in Fig. 5 and 6. Yield stress values were subtracted from shear stress readings and plotted against shear rate to determine power law constants. Yield stress values, power law

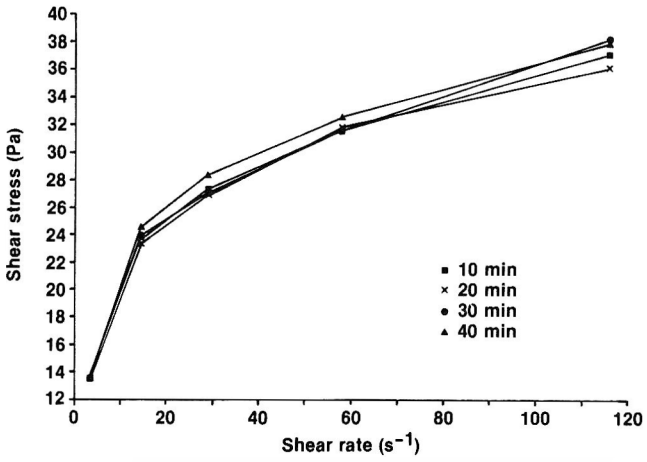

Fig. 3—Flow curves for yoghurt prepared from VAT heated milk at 85°C for 10 to 40 min.

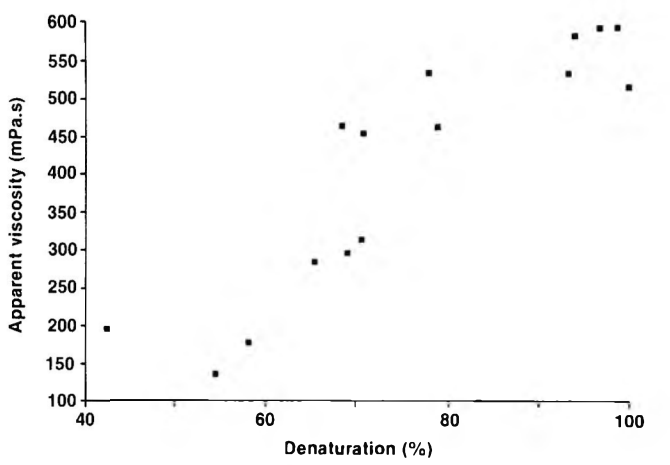

Fig. 4—Apparent viscosity of yoghurt versus degree of denaturation in corresponding heated milk (VAT, HTST and UHT).

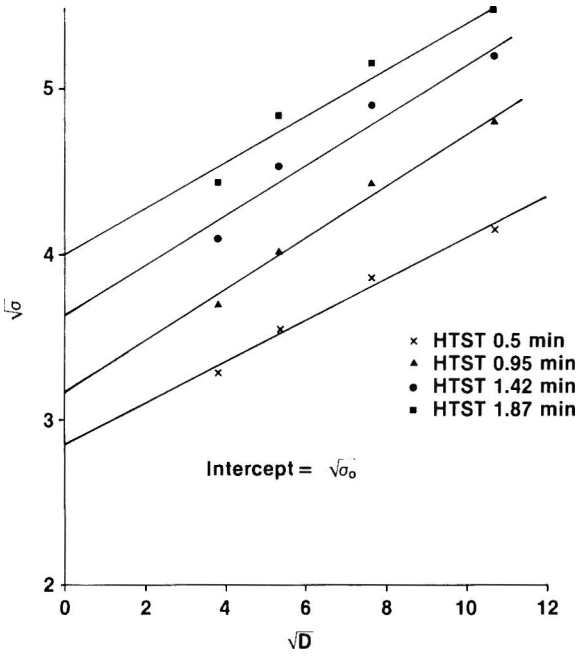

Fig. 5—Casson plot for yoghurt prepared from HTST heated milk.

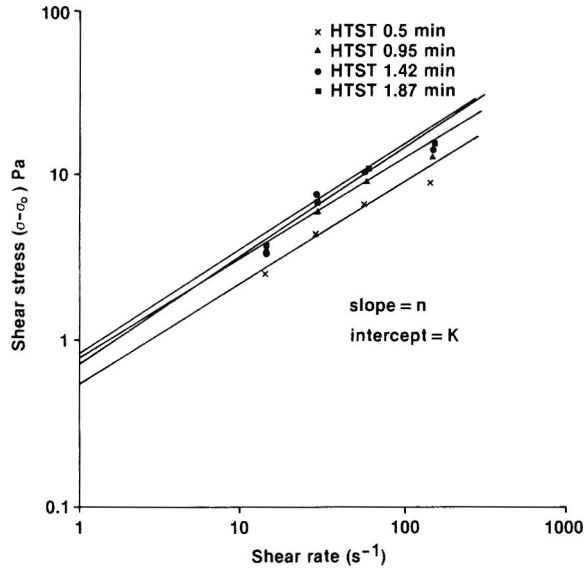

Fig. 6—Double logarithmic plot of shear stress versus shear rate for yoghurt prepared from HTST heated milk.

constants and R^2 values for Casson and log-lot plots are given in Table 2.

Flow behavior index, 'n', was not markedly affected by different heating systems with most values between 0.55 and 0.65. The flow behavior index measures the departure from Newtonian flow and results were consistent with pseudoplastic flow for which 'n' is less than 1. The consistency index 'K' was more affected by treatment, with VAT treatments being slightly greater than 1 while continuously heated treatments had values between 0.3 and 0.8.

Treatment effects were evident with yield stress values (Table 2) which followed the same trend as apparent viscosity values (Table 1). The correlation coefficient between apparent viscosity and yield stress was 0.987. Yield stress (σ_o) was not affected by residence time for VAT treatments, however σ_o was influenced by residence time in continuous heating systems. Simple correlations for yield stress versus residence time were 0.99 and 0.74 for HTST and UHT systems, respectively.

The close correlation between apparent viscosity and yield stress suggests that both parameters are influenced by the same properties. The presence of a yield stress value denotes a minimal stress which must be exceeded prior to flow occurring due to shear. This has been interpreted as the existence of a network structure, the bonds of which must be broken to allow flow (Sone, 1972). If the gel structure is considered to be composed of reversible and irreversible types of bonds, then it appears that only reversible types of bonds are broken with extended shearing. This implies that residual viscosity after shearing and yield stress are proportional to the number of irreversible bond sites in the gel structure.

Shear rate and time dependency

Time dependent behavior for HTST treatments is shown in Fig. 7. Total reduction in apparent viscosity due to shear and time are summarized in Table 3 for all treatments. All treatments exhibited similar reduction in apparent viscosity in both shear thinning and time dependent experiments. Reduction in apparent viscosity due to increasing shear rate was about 90 to 92% whereas reduction in apparent viscosity over a 10 min period at shear rate of 57.7 sec^{-1} ranged from 40–60%.

Thixotropic behavior has been defined as an isothermal, reversible sol-gel transformation (deMan, 1976). Labropoulos et al. (1981b) reported a similar decrease in apparent viscosity

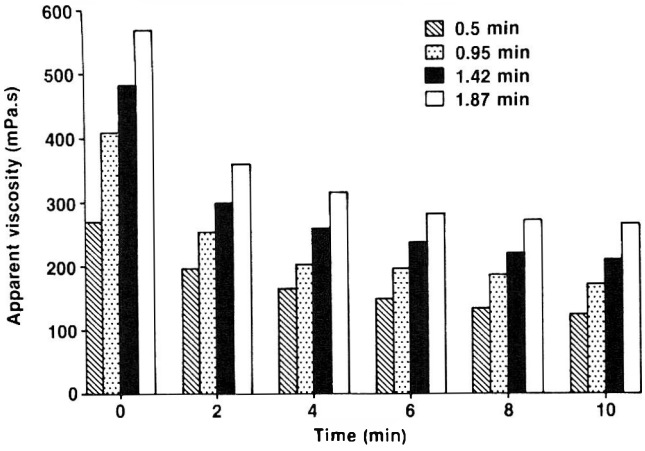

Fig. 7—Apparent viscosity as a function of time at constant shear rate (D = 57.6 sec^{-1}) for yoghurt prepared from HTST heated milk.

Table 1—Apparent viscosity[a] and pH[a,b] values for yoghurt prepared from HTST, UHT, VAT heated and unheated milk (Shear rate = 57.7 sec^{-1})

Treatment[c]	Apparent viscosity mPa.sec	pH
VAT 10 min	550.7	4.16
VAT 20 min	553.8	4.25
VAT 30 min	550.1	4.20
VAT 40 min	567.9	4.29
HTST 0.5 min	260.6	4.21
HTST 0.95 min	342.9	4.22
HTST 1.42 min	417.5	4.21
HTST 1.87 min	461.6	4.26
UHT 2 sec	170.2	4.15
UHT 4 sec	343.7	4.15
UHT 6 sec	298.8	4.22
UHT 8 sec	333.1	4.19
Unheated	197.7	4.20

[a] n = 3
[b] day 1 pH
[c] HTST = high temperature-short time heating at 98°C; UHT = ultra-high-temperature heating at 140°C; VAT = vat heating at 85°C.

with time of shear for yoghurt from UHT and VAT (batch) heated milk. That behavior was designated as thixotropic and explained by syneresis occurring with shearing. In view of the definition of thixotropic behavior given above (deMan, 1976), it does not seem appropriate to describe such behavior as thixotropic. It is unlikely, in our opinion, that the original apparent viscosity of yoghurt samples would be regained on standing. Rather, a partial recovery of structure is more likely which could be termed as pseudothixotropic (Rasic and Kurmann, 1978). Explanation in molecular terms is given as a breakdown of larger aggregates leading to a reduced effective radius. Partial reformation of gel structure would be due to aggregates coming together as the particles realign themselves.

CONCLUSIONS

TYPE OF HEATING SYSTEM for milk and residence time proved to be important variables affecting the apparent viscosity of yoghurt made from such milk. Rheological constants were less affected by treatment effects. The highest apparent viscosity was achieved by VAT treatments, and was almost equalled by yoghurt from HTST milk held for 1.87 min. Yoghurt from UHT heated milk gave markedly reduced apparent viscosity and, thus, would be of little interest to yoghurt processors as a viable alternative to batch processing systems. UHT treatments may, however, warrant further investigation for the manufacture of yoghurt beverages. Rheological constants provided are of interest in the design of processing systems for the manufacture of stirred yoghurt.

Table 2—Power law constants and R^2 values for yoghurt prepared from different heat-treated milks[a]

Treatment[b]	σ_o Pa	n	K	Casson R^2 %	Power law R^2 %
VAT 10 min	18.14	0.5827	1.248	99.2	99.3
VAT 20 min	18.07	0.5930	1.162	97.6	98.4
VAT 30 min	17.69	0.5670	1.405	99.9	99.9
VAT 40 min	19.14	0.5850	1.230	98.8	98.8
HTST 0.5 min	8.27	0.6022	0.551	97.6	98.0
HTST 0.95 min	9.99	0.6060	0.778	97.9	98.6
HTST 1.42 min	13.27	0.6441	0.831	95.8	95.3
HTST 1.87 min	16.01	0.6142	0.831	95.8	95.3
UHT 2 sec	4.31	0.6972	0.316	89.5	87.9
UHT 4 sec	8.94	0.6190	0.815	97.3	98.4
UHT 6 sec	9.12	0.5725	0.811	99.8	99.8
UHT 8 sec	8.73	0.6120	0.799	97.5	98.6
Unheated	3.51	0.6537	0.500	97.0	98.4

[a] σ_o = yield stress; n = flow behavior index; K = consistency index.
[b] VAT = vat heating at 85°C; HTST = high temperature-short time heating at 98°C; UHT = ultra-high-temperature heating at 140°C.

Table 3—Percent reduction in apparent viscosity of yoghurt as a function of increasing shear and time at constant shear

Treatment[a]	Reduction due to increasing shear[b] %	Reduction due to time at constant shear[c] %
VAT 10 min	91.3	50.7
VAT 20 min	91.6	50.8
VAT 30 min	91.1	50.6
VAT 40 min	91.3	48.4
HTST 0.5 min	91.6	53.9
HTST 0.95 min	91.1	58.4
HTST 1.42 min	92.3	56.7
HTST 1.87 min	92.6	53.6
UHT 2 sec	90.6	50.8
UHT 4 sec	91.7	53.3
UHT 6 sec	91.8	41.0
UHT 8 sec	92.3	49.9
Unheated	88.5	49.5

[a] VAT = vat heating at 85°C; HTST = high temperature-short time heating at 98°C; UHT = ultra-high-temperature heating at 140°C.
[b] Difference between apparent viscosity at D = 3.6 and D = 115.2 sec^{-1} expressed as % of apparent viscosity at D = 3.6 sec^{-1}.
[c] Difference between apparent viscosity at time = 0 and time = 10 min expressed as % of apparent viscosity at time = 0.

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Effect of Sweeteners and Stabilizers on Selected Sensory Attributes and Shelf Life of Ice Cream

S. A. WITTINGER and D. E. SMITH

ABSTRACT

Guar and locust bean gums were combined with sucrose, 36 DE corn syrup and/or 42 high fructose corn syrup (HFCS) in a minimum fat (10%) ice cream formulation. A total of 27 combinations were evaluated for sweetness, iciness, chewiness and vanilla intensity. Replacing 50% of the sucrose with 42 HFCS did not significantly affect sweetness or vanilla intensity. Chewiness was enhanced when high corn syrup levels were combined with a high guar to locust bean gum ratio. Storage study data showed that ice creams containing high levels of HFCS became objectionably icy earlier than ice creams containing low levels or no HFCS. With a high guar to locust bean gum ratio ice creams became both detectably and objectionably icy sooner than if a low ratio of these gums was used.

INTRODUCTION

THE EFFECT of various sweeteners and stabilizers on ice cream mix properties such as flavor, sweetness, texture, finished product quality, and storage stability continues to be an area of extensive investigation (Cottrell et al., 1979, 1980; Elfak et al., 1977, 1978, 1979a, 1979b, 1980; Eopechino and Leeder, 1967a, 1967b, 1969, 1970; Keeney, 1962, 1963). For many years the ice cream industry has used a variety of corn-derived sweeteners in combination with sucrose. Currently, use of corn sweeteners is increasing due to lower cost and functional advantages over sucrose. Lower dextrose equivalent corn syrups, which contain a significant number of long-chain dextrose polymers, are reported to produce a smoother ice cream, increase resistance to heat shock, and improve meltdown (Glazier and Mack, 1941; Keeney, 1962). High fructose corn syrups which contain a large quantity of monosaccharides are reported to enhance flavors and provide sweetness comparable to sucrose but at a lower cost (Langrange, 1979).

Numerous stabilizers have also been used in ice cream, primarily to inhibit ice crystal growth in product during distribution and storage (Arbuckle, 1981). Control of ice crystal formation has been attributed to the stabilizer's ability to reduce migration of free water in ice cream. Combinations and concentrations of stabilizers used in ice cream generally have been arrived at empirically.

As important as these two classes of ingredients are in ice cream, only limited data can be found concerning their interactions. A systematic evaluation of the effect of sweetener-stabilizer combinations on ice cream quality will give a better understanding of their interactions in ice cream. This research was undertaken to study the interactions of commonly used sweeteners and stabilizers on sensory attributes and shelf life of ice cream.

MATERIALS & METHODS

Mix formulation and processing

A mixture design (Cornell, 1981) was used to determine the effect of sweetener-stabilizer interactions on ice cream quality. The three sweeteners chosen—sucrose, 36 DE corn syrup (36 DE CS), and 42 high fructose corn syrup (42 HFCS)—were combined in nine different ratios as seen in Table 1. Each of these nine mixes was made with three different ratios (75:25, 50:50, 25:75) of guar gum to locust bean gum, giving a total of 27 different mixes. Mix 3 with a 50:50 ratio of stabilizers was produced seven times to determine variability in processing. Mixes were processed in random order.

Table 1—Combinations of sweeteners used for making ice cream mix

	% of Sweetener		
Mix	Sucrose	36 DE CS[a]	42 HFCS[b]
1	20	30	50
2	45	30	25
3	70	30	0
4	10	40	50
5	35	40	25
6	60	40	0
7	0	50	50
8	25	50	25
9	50	50	0

[a] 36 DE CS = 36 dextrose equivalent corn syrup.
[b] 42 HFCS = 42 high fructose corn syrup.

One hundred fifty pound batches of each ice cream mix were prepared by using 10% butterfat, 10% milk solids not fat, 17% sweetener solids (at 9 ratios mentioned above), 0.15% guar/locust bean gum (Continental Colloids), 0.015% carrageenan (Continental Coloids), 0.10% mono and diglycerides (Continental Colloids) and 62.74% water. Mixes were batch-pasteurized (68.3°C for 30 min), passed through a double stage homogenizer (2000 psig first stage and 500 psig second stage, respectively), and cooled to 5°C. Following aging for 4 hr a category II vanilla (Northville Flavor Co.) was added to the mixes. Mixes were frozen in a Cherry Burrell continuous freezer to a draw temperature of -4.6 ± 0.2°C and an overrun of 100% and dispensed in half-gallon rectangular cartons. Ice cream was hardened to -26°C and stored at this temperature until evaluated. Butterfat and total solids of each mix were determined in duplicate using the Mojonnier fat test described by Atherton and Newlander (1977) and oven solids method, AOAC (1980), respectively.

Sensory evaluation

Fresh product evaluation. Fourteen subjects (8 females and 6 males ages 22 to 35, all with prior ice cream judging experience) evaluated sweetness, iciness, chewiness and vanilla intensity of the ice creams. Before test samples of ice cream were evaluated, training and practice sessions were conducted for panelists. In practice sessions subjects discussed terms to be used and evaluated samples displaying various degrees of attributes to be evaluated. Twenty-four hours prior to evaluation, samples were removed from the -26°C freezer and tempered in a -15°C freezer. Samples were scooped into insulated serving trays fitted with 2-oz portion cups. Scooping was done in a 5°C cooler, and covered sample trays were held at -15°C until evaluation.

At each evaluation session, panelists evaluated one of the four sensory attributes of 12 samples of ice cream. These samples were three different gum ratios and sweetener combinations of mixes 1, 3, 7, and 9. Panelists evaluated samples using the unstructured category scaling technique described by Stone et al. (1974). The scale was 140 mm in length with 0 mm equal to lowest intensity and 140 mm equal to highest intensity of the attribute being evaluated. Each judge tasted samples in a different random order. Descriptors used were: not sweet to extremely sweet, no vanilla flavor to extreme vanilla flavor, not chewy to extremely chewy and not icy to extremely ice. Evaluations were conducted in individually lighted booths. Judges were presented

Authors Wittinger and Smith are with the Dept. of Food Science & Nutrition, Univ. of Minnesota, 1334 Eckles Avenue, St. Paul, MN 55108.

with spit cups, water, napkins, spoons, response forms and pencils and were asked to rinse their mouths with water between samples.

Data were analyzed using parametric analysis of variance. Where appropriate, multiple comparison tests were performed using Student-Newman-Keul's test (Steel and Torrie, 1980).

A second series of evaluations was conducted using all nine sweetener combinations containing the 50:50 ratio of guar gum to locust bean gum. For these evaluations, subjects received two trays of nine ice cream samples each. One tray was evaluated for vanilla intensity and the other for sweetness intensity.

Stored product evaluation. Development of iciness in ice creams was evaluated over a ten week storage period using a paired comparison technique (Amerine et al., 1965). The same 14 judges determined iciness and acceptability of stored samples. Ice creams were stored in a supermarket-type frost/defrost freezer which cycled from −9.4° to −15°C. In the freezer, sets of samples were systematically rotated each week. However, within a set, ice creams were randomly rotated. The freezer was kept full and its temperature was monitored throughout the study. The reference sample was mix 3 containing 50:50 ratio of stabilizers. This sample was stored at −26°C throughout the study and at the end of the study was judged to be free of iciness by the University of Minnesota Dairy Product Judging Team. Reference and test samples of ice creams were equilibrated to the same temperature (−15°C) 24 hr prior to evaluation. Initially, at each session, judges were served 27 pairs of ice creams. Their task was first to determine which sample in the pair was more icy, and second whether this degree of iciness was objectionable. Each panelist tasted samples in a different random order.

Analysis of data

Criteria set to compare ice creams were:

Detectably icy: if > 75% of the panelists chose the stored sample as being more icy than the reference for two consecutive weeks. The sample was assigned the first of the two consecutive weeks as the onset of iciness.

Objectionably icy: if >50% of the panelists rated the stored sample as objectionably ice for two consecutive weeks. The sample was assigned the first of the two consecutive weeks as the end of shelf life.

Data from paired comparison of iciness were used as an estimate of time to reach onset of iciness and were plotted on log-probability paper (Guadagni et al. 1975). Time when 75% of the panelists detected iciness in the samples was selected as the onset of iciness value using the following equation:

$$Pc = (Po - 50) * 2 \quad (1)$$

Pc is the probability value plotted on the X axis vs time on the Y axis. Po is the percentage of panelists identifying a difference. Paired comparison values from sensory evaluation panels were compared to onset of iciness value using regression analysis.

Weibull Hazard Analysis (Gacula and Kubala, 1975) was used to estimate mean values of "acceptable" shelf life. A calculated "% cumulative hazard" value based on number of panelists who found the ice cream sample objectionable is plotted on appropriate hazard paper (Team Electronics) versus age (weeks) to failure on the Y-axis. A straight line relationship is obtained if data conform to the Weibull distribution. Based on this distribution, the 50th percentile coincides with mean time of failure. This mean value is obtained by drawing a line from the intersection of the 50th point on the probability scale and the fitted line to the ordinate axis. Estimated times to failure, based on the hazard plotting values, were compared to sensory judgments of objectionable iciness of each ice cream by regression analysis.

RESULTS & DISCUSSION

Fresh product evaluation

Results of analysis of variance of sweetness intensity using combinations of four sweeteners and three stabilizers indicated that stabilizer ratios did not affect sweetness values [$p = 0.561$, $F(3, 143) = 0.58$]. However, as shown in Table 2, significant differences in sweetness intensity did exist among of the sweetener combinations [$p < 0.001$, $F(3, 143) = 6.7$]. Mixes 1 and 3, which contained CS at 30% of total sweetener solids and HFCS at 50% and 0% respectively were not different in sweetness from one another but were significantly sweeter than mixes 7 and 9, which contained CS at 50% of total sweetener solids and HFCS at 50% and 0% respectively. However, mixes 7 and 9 were not significantly different from each other in sweetness. Thus, substituting up to 50% HFCS for sucrose at a given level of corn syrup did not significantly affect sweetness, but increasing the level of coru syrup decreased sweetness.

Table 2—Sensory evaluation of sweetness and vanilla intensity for selected ice cream samples

Mix#	Sweetener Combination Sucrose:CS:HFCS[b]	Sweetness Intensity[a]	Vanilla Intensity[a]
1	20:30:50	75.0[c]	64.2[c]
3	70:30:0	71.8[c]	61.5[c]
7	0:50:50	45.9[d]	43.6[d]
9	50:50:0	53.3[d]	51.2[d]

[a] Means of 14 evaluations. Lower values indicate less intense sweetness or vanilla. Values are mm on a 140 mm unstructured category scale.
[b] CS = 36 dextrose equivalent corn syrup; HFCS = 42 high fructose corn syrup.
[c,d] Means within a column not followed by a common superscript are significantly different at $\alpha = 0.5$.

Table 3—Sensory evaluation of sweetness and vanilla intensity for ice creams with 9 sweetener combinations and stabilized with a 50:50 ratio of guar to locust bean gum

Mix#	Sweetener combination Sucrose:CS:HFCS[a]	Sweetness intensity[b]	Vanilla intensity[b]
1	20:30:50	70.7[cd]	50.6[cd]
2	45:30:25	72.8[c]	54.8[c]
3	70:30:0	75.3[c]	57.0[c]
4	10:40:50	65.5[cde]	38.1[cd]
5	35:40:25	62.5[cde]	46.0[cd]
6	60:40:0	48.7[def]	50.7[cd]
7	0:50:50	30.1[f]	29.0[d]
8	25:50:25	47.8[def]	36.5[cd]
9	50:50:0	42.6[ef]	42.1[cd]

[a] CS = 36 dextrose equivalent corn syrup; HFCS = 42 high fructose corn syrup.
[b] Means of 14 evaluations; (lower values indicates less intense sweetness or vanilla. Values are mm on a 140 mm unstructured category scale.)
[cdef] Means within a column not followed by a common superscript are significantly different at $\alpha = 0.05$.

Because stabilizers did not significantly affect sweetness intensity, all nine sweetener combinations at one stabilizer ratio (50:50 ratio of guar to locust bean gum) were evaluated for sweetness intensity. Significant differences in mean values (Table 3) were observed. A trend of decreasing sweetness values with increasing corn symp was apparent. This result agrees with data of Wolfmeyer (1963) that lack of sweetening power of low D.E. (dextrose equivalency) corn syrups implied lower total sweetness in ice cream with increased usage levels.

For a given percentage of corn syrup, % HFCS (25 or 50%) that replaced sucrose did not change perceived sweetness intensity ($p < 0.001$). This result substantiates claims of Robinson (1975) and of Hanover (1983) that comparable sweetness can be achieved in ice cream when 50% of the sucrose is replaced by 42 HFCS.

Analysis of variance data of combinations studies show stabilizer ratios did not significantly affect vanilla intensity [$p = 0.256$, $F(2, 143) = 10.11$]. Average means for three stabilizer combinations are presented in Table 2. Sweetener combinations altered vanilla intensity [$p < 0.001$, $F(3, 143) = 1.38$]. Products with a lower percentage of corn syrup (30%) had significantly higher vanilla intensity means than ice creams containing high corn syrup levels (50%). This confirms results of Windlan and Sheuring (1952). Lindamood (1967) also concluded vanilla flavor was partially masked with addition of corn syrup to ice cream. Also, at a given percentage of corn syrup (30 or 50%), vanilla intensity was not significantly altered by presence or absence of HFCS. Thus 42 HFCS did not alter vanilla intensity of ice cream. When all nine sweetener combinations (with 50:50 stabilizer ratio) were compared trends of lower vanilla intensity with increased percentage of corn

Table 4—Comparison of weeks to iciness of stored ice cream samples as determined by sensory panel (paired comparison test) and weeks predicted by using probability plotting

Mix#	Sweetener combination Sucrose:CS:HFCS[a]	Guar:Locust bean gum ratio 75:25 PC[b]	75:25 PP[c]	50:50 PC	50:50 PP	25:75 PC	25:75 PP
1	20:30:50	1	1.6	3	2.7	2	1.7
2	45:30:25	1	1.0	2	1.8	4	2.8
3	70:30:0	0	0	2	1.8	2	2.0
4	10:40:50	0	<1	2	2.4	3	2.0
5	35:40:25	2	2.6	2	2.0	3	2.0
6	60:40:0	2	1.4	2	1.2	4	3.0
7	0:50:50	1	<1	2	1.6	2	2.0
8	25:50:25	1	<1	1	<1	3	2.4
9	50:50:0	2	1.8	1	2.2	2	1.8
	MEAN	1.1	1.1	1.9	1.8	2.7	2.2

[a] CS = 36 dextrose equivalent corn syrup; HFCS = 42 high fructose corn syrup.
[b] PC = time for >75% of panelists to detect iciness in a paired comparison test.
[c] PP = 75 percentile based on probability paper as in Fig. 2 of the same data as for the PC analysis.

syrup and no change in vanilla intensity with substitution of HFCS for sucrose were again observed (Table 3).

Analysis of variance revealed degree of chewiness of the 12 ice cream samples was significantly affected by sweetener-stabilizer interactions [$p = 0.017$, $F(6, 143) = 2.68$] (Fig. 1). Existence of an interaction is not surprising since previous studies had concluded that corn syrups, especially low D. E. syrups, increased chewiness and might cause gumminess in ice cream (Keeney, 1962; Tremple, 1964). Stabilizers have also been reported to increase chewiness of ice cream (Arbuckle, 1981; Bayer, 1965).

Of four sweetener combinations examined, only mix 1 showed increased chewiness as amount of locust bean gum was increased; all others showed increased chewiness with increased levels of guar gum, Mix 9, which contained a high level of corn syrup (50%) and no HFCS, had a pronounced increase in perceived chewiness with increasing guar gum levels. High corn syrup levels in combination with high guar gum levels interacted to enhance chewiness of ice cream, while high corn syrup levels combined with high locust bean gum levels did not show such an effect. For a given corn syrup level (30 or 50%), replacement of sucrose with 42 HFCS lowered mean chewiness values. This may result from differences in freezing points: 42 HFCS lowered the freezing point of ice cream more than sucrose (Wittinger, 1985). Final texture is thus perceived as softer or "less chewy" than ice cream made with sucrose.

Analysis of variance of data for degree of initial iciness of 12 ice cream samples showed significant sweetener-stabilizer interactions [$p < 0.001$, $F(6, 143) = 5.28$]. This is consistent with ice cream literature indicating stabilizers and corn syrups aided in controlling ice crystal formation (Arbuckle, 1981). All means for initial iciness were relatively low (12-25 mm) and not significantly different from one another ($\alpha = 0.05$), implying good initial textural quality.

Stored product evaluation

All ice creams became detectably icier than the reference in 0 to 4 weeks and unacceptably icy in 3 to 10 weeks. Table 4 shows calculated time to onset of iciness for each ice cream based on paired comparison evaluation and estimated values from probability plotting technique. A typical plot of the use of probability statistics to estimate development of iciness is presented in Fig. 2. Linear regression of time to detectable iciness from raw data versus analysis by probability plotting gave a correlation coefficient of 0.71. Fig. 3 contains a typical Weibull Hazard plot of iciness data showing accumulated Hazard value (h) plotted vs failure time. Table 5 shows comparison of mean time to failure of each ice cream combination based on objectionable iciness from sensory evaluation versus estimated weeks from the Weibull Hazard Plot. Linear regression

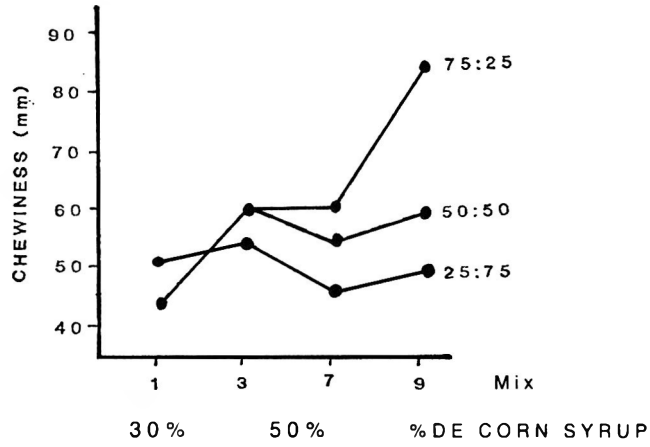

Fig. 1—Effect of percentage 36 DE corn syrup of the total sweetener solids on the sensory scores for chewiness (mm on the unstructured category scale) for ice cream samples containing three ratios (75:25, 50:50, 25:75) of guar:locust bean gum.

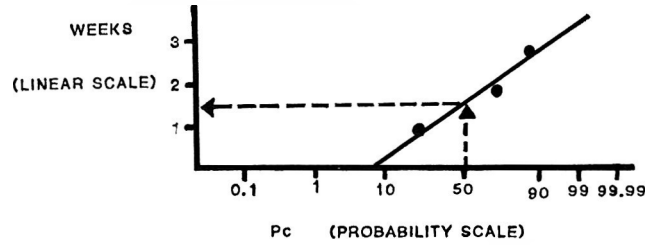

Fig. 2—Representative plot of the use of probability statistics for estimating the onset of iciness for a stored ice cream sample (a Pc value of 50 represents the time (weeks) where 75% of the panelists detect a difference in iciness between the stored sample and control sample.

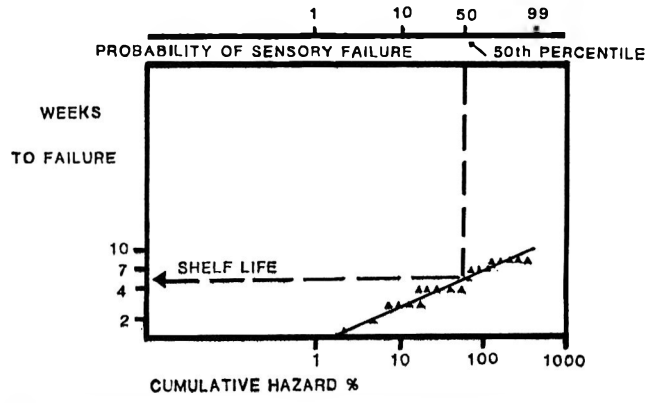

Fig. 3—Representative plot of the use of Weibull Hazard Analysis for estimating shelf life of ice cream (50th percentile for probability of sensory failure represents mean time (weeks) to failure for a stored ice cream sample).

of these values gave a correlation coefficient of 0.83. Based on correlation coefficients and mean values (storage lives averaged over sweeteners), both probability plotting and Weibull Hazard Analysis appear appropriate for analysis of storage data of ice creams. Correlation coefficients for each type of plotting could have been improved with more frequent sensory evaluations; plot values can be determined to the nearest day, whereas sensory judgments were determined to the nearest week. The straight line relationship obtained for each Weibull plot can also be used to predict probability of failure, i.e., percentage of products that are objectionable, at any week of storage, which might be useful from a marketing standpoint. One, however, must be careful when generalizing from small group data to the general population (Pangborn, 1980).

Table 5—Comparison of weeks for mean time to failure in stored ice cream samples based on iciness determined by sensory panel evaluation and weeks to failure predicted by Weibull Hazard analysis

Mix#	Sweetener combinations Sucrose:CS:HFCS[a]	Guar:Locust bean ratio 75:25 U[b]	75:25 W[c]	50:50 U	50:50 W	25:75 U	25:75 W
1	20:30:50	4	4.2	7	5.6	3	4.2
2	45:30:25	6	4.4	9	6.5	4	4.4
3	70:30:0	5	5.2	8	7.6	11	5.9
4	10:40:50	4	3.9	8	7.6	5	4.8
5	35:40:25	8	7.0	5	5.0	6	5.2
6	60:40:0	5	4.0	9	6.0	9	6.7
7	0:50:50	4	3.6	3	3.5	5	4.0
8	25:50:25	6	5.0	6	5.4	4	4.5
9	50:50:0	5	5.2	9	7.5	6	5.4
	Mean	5.2	4.7	6.7	5.6	5.9	5.0

[a] CS = 36 dextrose equivalent corn syrup; HFCS = 42 high fructose corn syrup.
[b] U = >50 percentile — based on sensory data.
[c] W = 50th percentile — based on Weibull Hazard Analysis.

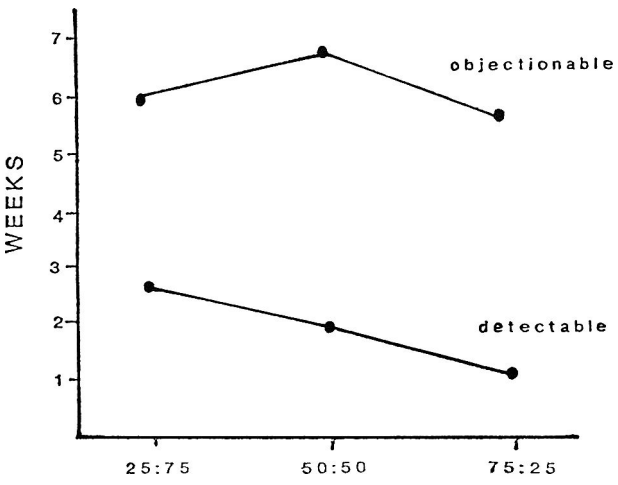

Fig. 4—Effect of guar:locust bean gum ratio on time (weeks) for ice creams to become detectably icy or objectionably icy (each point is averaged over the 9 sweetener combinations).

On the average, ice creams containing high levels of guar gum were detectably and unacceptably icy sooner than ice creams containing locust bean gum based on sensory data (Fig. 4). Data also indicate that higher usage levels of locust bean gum versus guar gum give the ice cream greater stability against ice crystal growth. This greater stability may be due to interaction of locust bean gum with itself or with carrageenan. This interaction increases the effective volume of the stabilizing particle (Blanchard and Mitchell, 1979), resulting in an increase in viscosity of the aqueous portion of the product. Because of this increase in viscosity, rate of ice crystal growth is slower. Guar gum has a lower interaction coefficient with like particles (Elfak et al., 1977) and does not complex as strongly with carrageenan as does locust bean gum (Blanchard and Mitchell 1979). Furthermore, during freeze-thaw conditions, locust bean gum shows a greater degree of association than does guar gum (Blanchard and Mitchell, 1979). This locust bean gum trend was not consistent for all ice creams, however, since sweeteners also play a role in storage stability. An example of this is seen with ice creams containing 42 HFCS as 25% of the total sweetener solids, where higher guar gum levels (either a 50:50 or 75:25 ratio of guar to locust bean) gum actually increased stability against iciness development.

Ice creams containing 50% 42 HFCS had the poorest storage stability for all stabilizer ratios, while ice creams containing 0% 42 HFCS had the best storage stability for all stabilizer ratios. These trends can be related partially to freezing point differences of the mixes (mixes high in 42 HFCS have lower freezing points). The higher the initial freezing point of a mix, the more water frozen at a given temperature. This is important for two reasons. First, mixes with high freezing points have less water available for participation in ice crystal growth which will occur during freeze-thaw cycles encountered in storage. Second, and more important, mixes with less free water will have a more viscous aqueous portion resulting in a reduced rate of ice crystal growth. However, freezing points are not the sole factor in determining shelf life of ice creams. As illustrated above, the particular combination of stabilizers affects the shelf life of ice cream, yet stabilizers have little effect on the freezing point of mixes. This effect on shelf life may be explained by properties of stabilizers. Degree of water binding of stabilizers is affected by combination of sweeteners present in the mix since both components are competing for the same water of hydration (Elfak et al., 1979a,b, 1980; Launay and Pasquet, 1982). Thus, sweetener-stabilizer combinations together influence shelf-life.

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Development of an Acceptable "Ice Cream" Possessing a Reduced Sodium Content

KEVIN M. ADDESSO and DICK H. KLEYN

ABSTRACT

Acceptable "ice cream" products possessing reduced sodium content were developed. Demineralized whey and demineralized whey protein concentrate were used individually and in various combinations to replace 15, 25, 50, 75, and 100% of the serum solids contributed by nonfat dry milk. Maximum reduction (63%) in sodium was achieved in one product containing 30.5 mg sodium/100g based upon comparison to a control containing 81.5 mg/100g. A descriptive product rating test was used to evaluate experimental products for appearance, flavor, body, texture, and acceptability. A student panel found all whey-containing experimental products to be acceptable when compared to the control sample, a high quality vanilla ice cream containing nonfat dry milk solids as the only source of serum solids.

INTRODUCTION

DEMINERALIZED WHEY PRODUCTS are promoted for special dietary food product applications and new whey protein concentrate (WPC) products are constantly being manufactured and marketed with special compositional and functionality attributes. Whey products with definitive sodium contents are available (Andres, 1982a,b,c).

The amount of whey solids used to apply serum solids in an ice cream is legally limited to 25% replacement of serum solids (FDA, 1978). Nilson (1975) demonstrated that as much as 75% whey solids could be substituted successfully for NFDM. Coder and Parsons (1979) demonstrated that there was no difference in acceptability between a control vanilla ice cream containing NFDM and experimental products in which 100% of the NFDM had been replaced by either WPC or a blend of WPC and dry whole whey. Young et al. (1980) demonstrated that frozen desserts of acceptable sensory and physical properties could be produced successfully using neutralized, lactose-hydrolyzed, fluid cottage cheese whey as a major bulk-supplying ingredient. A quality vanilla ice cream has been formulated from ultrafiltered milk resulting in 75% less lactose, up to 50% more protein than usual and as much as 50% decrease in sodium (Anonymous, 1985). The purpose of our study was to determine the amount of sodium reduction achievable in developing an acceptable "ice cream" by replacing various amounts of NFDM with demineralized whey products.

MATERIALS & METHODS

Preparation of control and experimental products

A standard ice cream formulation was prepared from sweet butter, a very good source of fat with very low sodium content (80% fat, 1% MSNF), non-fat dry milk (97% MSNF) sucrose, water and a stabilizer-emulsifier, PGX-1 (Germantown Mfg. Co., Broomall, PA). This compound contained mono- and di-glycerides, polysorbate 80, guar gum and carrageenan. Nine experimental frozen dairy desserts were produced using the same ingredients except that both demineralized whey solids (Nutritek®900) and demineralized whey protein concentrate (Foretein®35) were used either individually or in various combinations to replace successive amounts of NFDM (Table 1).

Author Kleyn is with the Dept of Food Science, Rutgers Univ., N.J. Agricultural Experiment Station, New Brunswick, NJ 08903. Author Addesso, formerly affiliated with Rutgers Univ. is now with Thomas J. Lipton, Inc., 800 Sylvan Ave., Englewood Cliffs, NJ, 07632.

Table 1—Composition of serum solids in mixes

Sample	NFDM	NUTRITEK®900 demineralized whey	FORETEIN35® whey protein concentrate
	% (w/w)		
Control	100	—	—
2	85	15	- - - -
3	75	25	- - - -
*4	50	50	- - - -
*5	25	75	- - - -
6	75	15	10
*7	50	25	25
*8	25	37.5	37.5
*9	- - - -	50	50
*10	- - - -	25	75

* Treated with lactase

Table 2—Proximate composition and mineral contents of NFDM and demineralized whey products

	NFDM	FORETEIN 35® whey protein concentrate	NUTRITEK®900 demineralized whey
	% (w/w)		
*Protein	35.4	34.2	11.8
Lactose	52.3	54.1	79.0-85.0
Moisture	3.0	3.5	4.2-4.9
Fat	0.8	3.4	0.8-1.3
Ash	8.0	3.0	0.8-1.2
	mg/100g		
*Sodium	495.0 ± 10.6	40.0 ± 3.5	3.5 ± 1.4
*Potassium	1688.8 ± 26.5	1135.0 ± 7.1	213.3 ± 2.5
*Calcium	1226.3 ± 8.8	247.5 ± 0.0	107.0 ± 3.5
*Magnesium	113.8 ± 1.8	87.5 ± 0.0	27.8 ± 0.4

* Determined experimentally. Lactose, moisture, fat, and ash data supplied by Foremost-McKesson, Inc.

Nutritek®900 (Foremost-McKesson, Inc. San Francisco, CA) is a high quality sweet dairy whey with 90% of the minerals removed (Table 2). It has a bland, slightly sweet taste unlike regular whey solids which have a salty flavor. Foretein®35 (Foremost-McKesson, Inc., San Francisco, CA) is a high protein acid whey product neutralized by potassium hydroxide. Approximately 90% of the ash is removed as well as some lactose (Table 2). Significant concentrations of nutritionally important calciuim, magnesium and phosphorous are claimed to be retained in the product. It is a cream-colored powder with a bland flavor.

The control and nine experimental mixes were formulated to contain 11% fat, 10.50% serum solids, 15% sucrose and 0.25% stabilizer emulsifier. A liquid neutral beta-galactosidase from the yeast *Kluyveromyces fragilis* (NOVO Laboratories, Inc., Wilton, CT) was added to those mixes where whey products were substituted for 50% or more of the NFDM (mixes 4,5,7,8,9, and 10). The purpose of this step was to prevent lactose crystallization during frozen storage. The lactase enzyme was added during the aging of the mix at the rate of 3.0 mL/liter of mix and was allowed at least 12 hr at 5°C to react in order to achieve a degree of hydrolysis of approximately 80% (Stevenson, 1983).

Twenty-five pounds (ca. 10.5 liters) of each mix were prepared in 5-gal stainless steel shotgun cans. Mixes were batch-pasteurized in a hot water bath at 71°C for 30 min with constant stirring, homogenized in a two-stage homogenizer (Gaulin, Model #4030) with 2000 psi

applied at the first stage and 500 psi at the second. Mixes were then cooled quickly to 23°C in an ice bath, and then to 1.7°C by storing overnight in a cold room. Mixes were flavored with two-fold vanilla extract (Virginia Dare Extract Co., Brooklyn, NY) at the rate of 4 ml/liter and frozen in a batch-type freezer (Emery Thompson, Model #2HCF). Mixes were drawn off into half gallon containers when a 75% overrun was achieved. Products were then stored in a hardening room at −28.9°C until they were evaluated or sampled for analyses.

Sensory testing

An untrained panel consisting of thirty students enrolled in a sensory evaluation class was used to evaluate the nine frozen dairy desserts for appearance, flavor, body, texture, and acceptance. The control mix was formulated to produce an acceptable vanilla ice cream and was judged by the authors to possess ideal attributes (numerical score = 9-10) as described in *Judging Dairy Products* (Nelson and Trout, 1964).

Students were instructed to evaluate each of the attributes of the experimental frozen dairy desserts 2–10 using the control as a reference. Samples were tempered at 4°C and approximately 40g were scooped out into coded plain paper cups. Since the panelists were not specifically trained for judging ice cream, a brief discussion on the difference between body and texture of an ice cream was presented to them prior to each session.

The type of test used was a descriptive product rating test using the descriptors excellent, good, fair, poor and unacceptable. Each term was assigned a numerical value: 9 = excellent, 7 = good, 5 = fair, 3 = poor and 1 = unacceptable. Scores were then tabulated and the mean and standard deviation were reported for each attribute of every sample.

Panelists were also instructed to rank each product in terms of sweetness indicating whether it was more, less or the same in sweetness as compared to the control. They were also encouraged to make additional comments on the desserts.

No more than three samples and the control were presented to the panelists at any one session. Nine samples were presented during four sessions over a period of one month. The control and mixes 2–5 were prepared during the first week and the first two sessions to evaluate them were conducted during the second week. The same procedure was followed for samples 6-10 during the next two weeks. Samples 4 and 8 were prepared and presented to the panelists three weeks after the last session to obtain an indication of the panelists' reproducibility.

Chemical analyses

Atomic absorption spectrophotometry (Perkin-Elmer 290. Perkin-Elmer, Norwalk, CT) was used to determine sodium, as well as potassium, calcium and magnesium in the experimental mixes, commercial samples, NFDM and whey powders. Protein-free filtrates of samples were prepared and used for mineral analyses (Addesso, 1985). Samples of the unfrozen experimental mixes were used for chemical and mineral analyses. The sampling method for the frozen commercial desserts was according to the Association of Analytical Chemists (AOAC) Official Method of Analysis 16.283 (AOAC, 1980).

Protein content of the experimental mixes, frozen commercial samples, NFDM and whey powders was determined to duplicate by a Micro-Kjeldahl method adapted from the Macro-Kjeldahl method (AOAC, 1980). A modification for fluid mixes and dry powders was used (Addesso, 1985). Fat content of experimental mixes was determined in duplicate by the Gerber method (Case et al., 1985). Total solids content of the mixes were determined in duplicate by the Mojonnier method (Mojonnier Instruction Manual, 1922).

RESULTS & DISCUSSION

NFDM contained the greatest amounts of all four minerals followed by the demineralized WPC and demineralized whey (Table 2). The demineralized WPC contained proportionately more potassium than other minerals since it was neutralized with potassium hydroxide during processing.

Concentrations of sodium, potassium, calcium, and magnesium ranged from high levels of 81.5 ± 0.9, 175.5 ± 2.3, 123.5 ± 4.1, and 11.4 ± 0.2 mg/100g to low levels of 30.5 ± 0.5 mg/100g, 64.0 ± 2.6, 24.0 ± 2.3, and 5.3 ± 0.5 mg/100g, in the control and experimental mixes, respectively. Reductions (%) achieved in the sodium contents were significant (Table 3); reductions of approximately 63% being achieved in products 9 and 10. The greatest reduction in potassium occurred in product 5 which contained 25% NFDM and 75% Nutritek®900. The greatest reduction in calcium occurred in product 10, while the greatest reduction of magnesium occurred in product 9.

Table 3—Percent reduction in minerals achieved in experimental frozen desserts

Sample	Sodium	Potassium	Calcium	Magnesium
Control	—	—	—	—
2	3.7	12.3	14.6	10.5
3	12.9	14.3	19.4	15.8
4	25.8	38.8	38.1	34.2
5	43.9	63.5	59.8	47.4
6	16.6	8.3	17.4	7.9
7	31.3	25.4	40.5	36.8
8	37.4	41.6	57.1	41.2
9	62.3	59.3	78.1	53.5
10	62.6	44.4	80.6	41.9

Table 4—Mineral concentration of commercial frozen desserts

Sample	Sodium	Potassium	Calcium	Magnesium
	mg/100g			
1	61.0 ± 1.5	220.5 ± 4.0	152.5 ± 6.0	13.5 ± 0.5
2	62.0 ± 0.5	210.0 ± 2.5	136.5 ± 1.5	13.0 ± 0.0
3	75.0 ± 1.5	207.0 ± 4.0	110.5 ± 1.5	11.5 ± 0.5
4	80.0 ± 0.5	222.0 ± 1.5	110.5 ± 2.0	12.5 ± 0.5
5	84.5 ± 1.0	219.5 ± 1.0	108.0 ± 1.0	12.5 ± 0.5
*6	127.5 ± 1.5	264.5 ± 4.5	182.0 ± 2.0	21.5 ± 0.5
*7	83.5 ± 0.5	242.5 ± 1.0	134.5 ± 2.7	14.2 ± 3.4

* Samples 6 and 7 are ice milks, all others are ice cream products. All values are based on a mean value calculated from 5 replicates ± standard deviation.

Since the weight of a typical 8 fl oz serving of ice cream or ice milk may range from 130–175g depending upon the overrun (Posati and Orr, 1976), the sodium concentration(s) of the experimental frozen desserts would be expected to be 1.3 to 1.75 times greater per serving than per 100g. The sodium content of the control and each experimental product would have to be labeled to the nearest 5 mg increment according to the proposed labeling regulation pertaining to sodium (THRUST, 1984). The descriptive term "Low Sodium" may be used for any of these experimental products with the possible exception of the control since each would contain < 140 mg of sodium per serving. The descriptor "Very Low Sodium" (< 35 mg of sodium per serving) could be used for products 9 and 10 if a higher overrun was achieved and a typical 8 fl oz serving weighed 100g or less. A frozen dessert with a sodium content similar to that of experimental products 9 and 10 could possibly be labeled as a "Reduced Sodium" product if it were represented as a direct replacement for a dietetic ice milk product which contained four times the amount of sodium (THRUST, 1984).

One drawback in the use of demineralized whey products is that reduction of other valuable minerals such as calcium, potassium and magnesium also occurred. However, significant reductions in sodium can be achieved while minimizing reductions in calcium and potassium if proper selection of whey ingredients is followed. Mineral salts may also be added to the product. In terms of legal requirements, six of the products (4,5,7,8,9, and 10) would not meet the current federal standard of identity for ice cream which stipulates that no more than 25% of the milk solids-not-fat may be substituted with whey solids.

Levels of sodium, potassium, calcium, and magnesium (Table 4) in several commercial ice cream samples were determined in order to gain an idea of the range of concentrations of these minerals in ice cream. The product containing the lowest level of sodium did not necessarily contain the lowest levels in potassium, calcium, and magnesium. This reflects the many types and large variety of ingredients used in ice cream formulations. Sample 6, a dietetic ice milk product, contained

the highest levels of the four mienrals and did contain sodium chloride in its formulation. Four of the commercial products (3,4,5, and 7) contained a level of sodium similar to that of the control product formulated for this study. Experimental samples 9 and 10 contain less than half the amount of sodium than the commercial product with the lowest sodium content. The levels of potassium, calcium and magnesium were generally greater in the commercial products than for either the control or experimental products.

The sodium concentration of commercial samples 1 to 4 would be expressed to the nearest 5 mg increment and could have the descriptor "Low Sodium" appear on their labels. Samples 5,6, and 7 would be labeled to the nearest 5 mg or 10 mg increment depending upon the weight of the serving size and may only be labeled as "Low Sodium."

Sensory results

All of the experimental frozen dairy desserts were judged to be acceptable in all attributes as well as in the overall preference rating based upon evaluation of the mean scores (Table 5). All samples received ratings ranging from fair to good and no product received a score less than fair. Samples 9 and 10 did receive slightly lower ratings from several panelists who offered more detailed criticism. However, when the standard deviations(s) are considered, the samples were not significantly different from one another. No attempt was made to determine which of the nine experimental ice creams had the optimum flavor score relative to amount of reduction in sodium content. However, the scores indicate that an acceptable frozen dairy dessert can be made with 100% of the serum solids being contributed by demineralized whey products.

The authors, as well as some panelists, noticed that products 9 and 10 possessed a "whey-type" flavor when evaluated. Some panelists used descriptive terms such as "egg-like" or "almond-flavored" to describe this flavor. In addition, the color of products 9 and 10 was notably yellow but apparently the panelists did not judge this to be unacceptable. In fact, one panelist described product 10 as being "darker, a French vanilla color." The color of products 9 and 10 did make them appear as French vanilla products. This yellow color can most likely be attributed to the yellow-colored WPC (Foretein®35). One interesting observation made by the authors was that the "whey-like" flavor and yellow color of the products containing high levels of demineralized whey and/or WPC were not as pronounced immediately after freezing as they were after storage in the hardening room.

More than 50% of the panelists judged products 5, 9, and 10 to be sweeter than the control. These products contained both glucose and galactose due to the subsequent hydrolysis of lactose. These two monosaccharides together are sweeter than lactose. However, no conclusive statements could be made about sweetness since some products which also contained hydrolyzed lactose were not found to be sweeter (4, 7 and 8) than the control.

Another observation made by the authors was the notably softer consistency of those products containing hydrolyzed lactose. These products could be scooped easily immediately upon removal from the hardening room. Those products without hydrolyzed lactose were much firmer and harder to dip. This increased freezing point depression is caused by the greater concentration of lower molecular weight sugar molecules. Replacement of NFDM with demineralized whey and/or WPC at a level of 50% or more resulted in products with a notably lighter or thinner body. The bodies of products 9 and 10 were very weak, perhaps because these products may have had a slightly higher overrun as they whipped very easily and it was difficult to assure 75% overrun even though only first-drawn samples were used. No noticeable problems with texture were noted for the products. The addition of the beta-galactosidase enzyme prevented lactose crystallization in those products where 50% or more of the NFDM was replaced by demineralized whey and/or WPC.

The descriptive product rating test was designed to determine acceptability not the simultaneous comparison of the many variables present. This condition prevented the isolation of any one variable as being solely responsible for any major sensory defect or improvement. The extent of NFDM replacement by demineralized whey powders, the single type or mixture of the two used, as well as the presence of hydrolyzed lactose, could alone affect any one attribute. However, these effects may have been confounded. For example, the "whey-like" flavor of those products containing high levels of whey powder(s) may have been masked by the resultant increase in sweetness caused by the addition of lactase. A factorial design can be used to measure preference and determine what effect a specific ingredient or process might have on each attribute. This approach should be followed in any future research.

Results from the retest of samples 4 and 8 yielded similar scores which indicated the panel's ability to yield reproducible results.

Protein, fat and total solids

The control mix contained the highest percentage of protein since NFDM was the sole source of serum solids used. The average protein content of the experimental mixes was 2.86% and levels ranged from 2.04% where 75% of NFDM was replaced by Nutritek®900, to 3.28% where only 15% of the NFDM was replaced by Nutritek®900 (Table 6).

The mixes (2 to 5) in which Nutritek®900 was used to replace NFDM at levels of 25, 50 and 75% contained less protein than those (6 to 8) in which combinations of the two whey products were used in replacing the NFDM at the same levels. This is due to the higher protein content of Foretein®35 as compared to Nutritek®900.

Foretein®35 may be used to reduce the serum solids' contribution of sodium to the product without sacrificing a loss in protein since its protein content is similar to that of NFDM. However, since the Protein Efficiency Ratio of whey protein exceeds that of casein, Foretein®35 may be considered to be a more nutritional source of serum solids. The use of Nutritek®900 resulted in a greater reduction in the sodium level but protein was also significantly lowered.

The protein content of the whey mixes determined in this study are similar to those reported by Young et al. (1980) who

Table 5—Sensory data (mean score ± standard deviation) for experimental frozen desserts*

Sample #	Appearance	Flavor	Body	Texture	Acceptance
2	7.7 ± 1.1	6.9 ± 1.2	6.9 ± 1.4	6.9 ± 1.2	7.2 ± 0.6
3	7.7 ± 1.3	7.3 ± 1.4	6.9 ± 1.4	6.7 ± 1.4	7.1 ± 1.3
4	7.6 ± 1.3	6.9 ± 1.7	6.8 ± 1.6	7.0 ± 1.4	7.2 ± 1.3
5	7.6 ± 1.5	6.6 ± 1.5	6.7 ± 1.4	7.0 ± 1.3	6.7 ± 1.3
6	7.9 ± 1.1	7.2 ± 1.5	7.1 ± 1.3	6.9 ± 1.2	7.1 ± 1.2
7	7.5 ± 1.3	6.4 ± 1.4	6.6 ± 1.6	6.6 ± 1.5	6.5 ± 1.4
8	7.7 ± 1.1	7.2 ± 1.3	7.1 ± 1.3	7.0 ± 1.4	7.2 ± 0.8
9	7.6 ± 1.2	5.7 ± 1.8	6.6 ± 1.5	6.6 ± 1.6	6.5 ± 1.4
10	6.8 ± 1.7	6.0 ± 1.5	6.5 ± 1.7	6.6 ± 1.6	6.7 ± 1.3

* 9 = excellent; 7 = good; 5 = fair; 3 = poor; 1 = unacceptable.

Table 6—Protein content of experimental mixes

Mix Number	Protein (%)
Control	*3.38
2	3.28
3	3.19
4	2.53
5	2.04
6	3.25
7	3.05
8	2.80
9	2.58
10	3.05
	$\mu \pm \sigma$ = 2.86 ± 0.41%

* Not included in calculation of average protein content of whey-based products.

used 53% whey, by weight of the total mix, to achieve a 29.2% mean replacement of MSNF by whey solids and found the whey mixes to contain an average of 3.01% protein. Lowenstein et al. (1975) used hydrolyzed whey to replace 100% or 50% of the MSNF and found the protein content to be 1.81% or 3.11%, respectively. One survey of fifteen commercial ice creams revealed that protein ranged from 2.49–4.38%, with a mean of 3.41% (Kristoffersen and Miller, 1976). The average total solids content of the experimental products was 37.77% and the average fat content, 11.15%.

CONCLUSIONS

AN ACCEPTABLE "ice cream" possessing a maximum reduction of 63% in sodium content as compared to a control product has been manufactured by replacement of over 75% of the serum solids with demineralized whey solids. While ice cream is not considered to be a high sodium containing product, it is consumed in high amounts by many consumers wherein the level of sodium could become of concern to individuals on sodium-restricted diets.

This potential problem is of greatest concern for those individuals who consume low fat ice milk of dietetic desserts on a regular basis. These products generally contain higher levels of sodium as well as other minerals because of the increased usage levels of serum solids which are used to compensate for the loss in the total solids level. These low calorie dietetic desserts are marketed toward individuals concerned with weight reduction and who may possibly be on some type of sodium modified diet. For individuals on sodium restricted diets, the availability of alternative lower sodium food products would allow for their more frequent consumption and would also help to increase the variety of other foods allowed in their diets.

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Differentiating the Lectin Activity in Twenty-Four Cultivars of Dry Beans (*Phaseolus vulgaris* L.)

H. H. KOEHLER, HEDWIG E. HERRICK, and D. W. BURKE

ABSTRACT

Hemagglutination activity of 24 cultivars of dry beans (*Phaseolus vulgaris*) was estimated with rabbit, rat, bovine, and human erythrocytes. Net protein utilization (NPU) evaluated the nutritional toxicity to weanling rats of the beans fed at 10% protein. Nonprotein and 10%-casein control diets were included. SDS-polyacrylamide gel electrophoresis indicated lectin-containing beans. Beans evaluated (in order of *decreasing* lectin activity) were: Aurora, Sanilac, Royal Red, Red Kloud, Roza, Rufus, Harris, Viva, Fiesta, Black Turtle Soup, Chief, Hyden, UI-59, Sutter; (nontoxic): Blue Mountain, GN-1140, Holberg, Nodak, Olathe, Pindak, JM-126, NW-410, NW-590, UI-114. This study shows that combined hemagglutination tests and SDS-PAGE enable rapid screening of dry beans for toxic lectins.

INTRODUCTION

LECTINS (hemagglutinins) are glycoproteins which occur naturally in most types of dry beans (*Phaseolus vulgaris* L.). The toxicity of lectins in beans to animals, birds, and people has been reviewed by Coffey et al. (1985).

Criteria reported for determining lectin toxicity of raw beans are: (1) agglutination of animal red blood cells (Bender, 1983; Jaffe, 1980; Brown et al., 1981a, b; 1982a, b; Pusztai and Watt, 1974); (2) toxicity to animals in Net Protein Utilization (NPU), Protein Efficiency Ratio (PER), or other feeding trials (Pusztai et al., 1979b; Pusztai and Palmer, 1977; Evans et al., 1973; Grant et al., 1985; Williams et al., 1984/85; Rouanet et al., 1985; Pusztai et al., 1981a; Jayne-Williams and Burgess, 1974); and (3) specific lectin bands in the protein patterns separated by SDS-polyacrylamide gel electrophoresis (Pusztai et al., 1981a, b, 1982; Pusztai and Watt, 1974; Brown et al., 1981b).

Although Jaffe et al. (1972) reported that heating some types of beans at 80°C for 90 min failed to destroy their toxicity, Stein (1976) and Grant et al. (1982) found that toxic effects could be inactivated by boiling hydrated beans for at least ten minutes. Bender and Reaidi (1982) found that heating beans for 15–45 min at 80°C increased hemagglutination activity about 5X; hence partially cooked beans may be more toxic than those eaten raw. Toxicity of beans has not been a problem in the Unites States until recently, when the popularity of low-temperature cooking methods and of eating raw and "natural" foods focused attention on lectins in foodstuffs (Nachbar and Oppenheim, 1980; Noah et al., 1980; Bender and Reaidi, 1982), and on the need for adequate soaking and heat processing of beans and bean products (Jaffe, 1980; Grant et al., 1982; Thompson et al., 1983; Bender, 1983; Coffey et al., 1985). Lectin toxicity is much more prevalent in developing countries, where scarcity of fuel and lower boiling points of water may make sufficient cooking difficult (Korte, 1972; Silbernagel, 1984).

Authors Koehler and Herrick are with the Dept. of Food Science & Human Nutrition, Washington State Univ., Pullman, WA 99164-2032. Author Burke was Research Plant Pathologist, Vegetable Crops Production Research Unit, ARS-USDA, Irrigated Agriculture Research and Extension Center, Prosser, WA 99350.

Bean types appear to vary in the amount and toxicity of their lectins. Pusztai et al. (1979b; 1981b, 1982) studied both a Pinto (cv. UI-111) and a Great Northern cultivar which had low concentrations of lectin and were non-toxic to rats; however, they reported that Red Kidney and White Snap Beans tested had hemagglutinating action and were toxic to rats. Evans et al. (1973) studied a Navy Bean (cv. Sanilac) which was toxic. Nachbar and Oppenheim (1980) reported moderate to great lectin activity in eight types of *P. vulgaris* among 88 vegetables and fruits tested. Jaffe (1980) pointed out that bean-type designations (Pinto, Pink, Great Northern, etc.) and seed-coat color bear no relationship to presence of toxic lectins, and that lectin properties vary from cultivar to cultivar.

Jaffe et al. (1972) classified bean cvs into four groups according to specificity of their hemagglutination activity. Type A beans strongly agglutinated all RBC, Type B beans agglutinated all RBC except trypsin-treated bovine cells, Type C beans agglutinated only trypsinated bovine cells and pronase-treated rat cells, and Type D beans did not agglutinate any RBC.

Little is known about the lectin content of many of the dry bean cultivars grown commercially. Knowledge of the content and toxicity of bean lectins is needed for optimum utilization of beans as a valuable source of protein, especially in developing countries. The purpose of this study was to differentiate the lectin activity in twenty-four cultivars representing nine bean types grown commercially in the United States.

MATERIALS & METHODS

Beans

Twenty-four *Phaseolus vulgaris* cultivars representing nine types of beans (Table 1) were grown at Prosser or Othello, WA, by personnel of the Washington State University Irrigated Agriculture Research and Extension Center (IAREC). Dry beans were hand-sorted to ensure purity, and ground in a Udy Laboratory Mill to pass a 60-mesh screen.

Hemagglutination assay

Bean powder was extracted (50 mg powder per mL) with 0.04M $NaBO_3$ buffer, pH 8.0, for 16 hr at 0–5°C, and centrifuged for 1 hr at 40,000 × *g*. The supernatant was used for testing. Twenty-five microliters saline were put in wells of Cooke micro-titer plates and 25 μL bean extract supernatant were added to well #1 of each row. Serial dilution was accomplished from well #1 through 12 or 15 wells as needed (until no hemgglutination resulted), using 25 μL manually manipulated Cooke Micro-Dilutors (Dynatech Laboratories, Inc., Alexandria, VA). To each well were finally added 25 μL prepared, diluted red blood cells (RBC) (Pusztai and Watt, 1970). Plates were sealed with plastic wrap until examination after four hours and sixteen hours.

Rat and rabbit bloods were received from the WSU Laboratory Animal Resources Center (LARC); bovine blood was collected by the Department of Animal Sciences; human blood was drawn by technicians at Pullman Memorial Hospital. Each blood sample was collected into a pre-heparinized tube, diluted immediately (1 mL blood + 24 mL 0.9% NaCl), and stored at 1°C. Rabbit and human 0^+ RBC were used without further treatment. Rat RBC (diluted 1:25) were centrifuged, washed twice with saline, resuspended in saline to original volume, and incubated for 40 min at 25°C with 0.2 mg pre-dissolved pronase per 10 mL diluted RBC. After centrifuging and quadruple

Table 1—Type and genetic heritage of bean cultivars (Phaseolus vulgaris) *tested*

Cultivar	Type	Genetic background[a] (Originator)
Aurora	Small White (SW)	White mutant from black-bean cross (NY)
Chief	SW	CA SW × RM UI-35 (Stokely-Van Camp)
Hyden	Navy (N)	Aurora × Pinto UI-114 (USDA/WA/OR)
Sanilac	N	Radiation mutant from Michelete (MI)
Harris	Great Northern (GN)	GN Montana #5 × *P. acutifolius* Nebraska #1 selection #27 back-× GN 1140 (NE)
GN-1140	GN	Native beans GN UI-123 × Pinto #5 (1949; USDA/MT)
UI-59	GN	Unknown (1953; ID)
Roza	Pink (PK)	P65R1093 breeding line (br.ln.) × Sutter PK (USDA/WA)
Sutter	PK	Mutant from CA native PK beans (CA)
Viva	PK	65R-159 br.ln. × Sutter PK (USDA/WA)
Rufus	Red Mexican (RM)	RM UI-35 back-× P.I. 203958 (USDA/WA/ID)
Fiesta	Pinto (P)	Not publicized (patented cv of Idaho Seed Bean Co., Twin Falls)
Holberg	P	UI-114 × br.ln. 5R-568 × Viva PK (ND/WA/USDA)
Nodak	P	2R39-1 PK × Royal Red Kidney 3R187-1 × GN-1140 (ND/WA/ID/USDA)
Olathe	P	Complex incl. UI-114, Pinto #5, and a Red Kidney (patented cv. of Colorado State University)
Pindak	P	UI-114 × br.ln. 5R-568 × Japanese red-seeded bush bean (ND/USDA/WA)
JM-126	P	Nep II × NW-410 Pinto back-× NW-410
NW-410	P	UI-114 × Sutter PK (USDA/WA/ID/OR/CO)
NW-590	P	UI-114 × br.ln. 65R-1059 (USDA/WA/ID/OR)
UI-114	P	Old commercial cultivar (1960; ID)
Black Turtle	Black (B)	Old commercial cultivar
Red Kloud	Light Red Kidney (RK)	NY (Cornell)
Royal Red	RK	[br. ln. × RK] × br. ln. (USDA/WA)
Blue Mountain	Green-Pod Bush Snap Bean (French Bean)	Blue Lake, several snap bean br.ln., GN, SW, some *P. coccineus* (USDA/WA)

[a] Records of ARS-USDA/IAREC, Prosser, WA

washing with saline, rat cells were suspended to original volume in saline and stored at 1°C. Bovine blood was treated similarly, except that pre-dissolved trypsin was added (0.1 mg per 10 mL diluted RBC), and incubation was for 60 min at 25°C. Treated RBC were freshly prepared for each test.

Plates were examined microscopically by transferring the thoroughly mixed contents of each well to a hemacytometer grid. Degree of agglutination was estimated after 4 and 16 hr. Three replications reacted each bean powder with each of the four species of RBC.

Net protein utilization (NPU) evaluation

Twenty-day-old Sprague-Dawley rats from the WSU LARC colony were fed a stock diet for 10 days then divided into groups of four rats having equal total weights, according to the NPU procedure of Palmer et al. (1973) and Miller and Bender (1955). Each group was fed a test diet containing corn starch, dextrose, corn oil, minerals and vitamins plus protein from a test bean powder or casein at 10% (w/w) of the diet at the expense of the starch. A nonprotein-diet control group was included. The protein sources were not supplemented with individual amino acids. Each rat was weighed daily during the test period. Feces were collected from test day 3.

All diets, rat carcasses and feces were analyzed for moisture content and Kjeldahl nitrogen (AOAC, 1980). Carcasses were opened ventrally, freeze-dried, and ground to powder in a Braum Model KMM-20 coffee mill. Dried feces were pulverized in an Osterizer blender. NPU was calculated according to the following equation (Miller and Lachance, 1977):

$$\text{NPU} = \frac{\text{body N of test protein group} - \text{body N of protein-free group}}{\text{N intake of test group}} \times 100$$

Digestibility determinations

In vitro protein digestibility of the bean powders was estimated by the multienzyme technique of Hsu et al. (1977). Apparent digestibilities were determined *in vivo* during the NPU rat-feeding study as follows:

$$\text{Apparent digestibility} = \frac{\text{N in diet (g)} - \text{N in feces (g)}}{\text{N in diet (g)}} \times 100$$

Electrophoresis separation

SDS-polyacrylamide gel electrophoresis was performed using a Bio-Rad vertical slab gel apparatus, Model 220 (Richmond, CA). A discontinuous system employing a 10% separation gel overlayered with a 4% stacking gel was adapted from Laemmli (1970). Bean extracts were prepared with 0.01M SDS-phosphate buffer (12 mg/mL), pH 7.4. Ten microliters of extract were inserted in each of 11 wells, and 100-m.amp current was applied for 6½ hr. Gels were stained with Coomassie blue and destained with methanol:glacial acetic acid:water (5:2:13, v/v/v). Protein molecular weight standards used were egg albumin, mol. wt. 66,000 daltons; pepsin, 34,700 daltons; trypsinogen, 24,000 daltons; and Mark-VI (Sigma), six bands ranging from mol. wt. 66,000 daltons down to 14,300 daltons.

RESULTS

Hemagglutination activity

Degree of hemagglutination of untreated rabbit and human 0^- RBC, pronase-treated rat RBC, and trypsin-treated bovine RBC by the 24 bean powder extracts is shown in Table 2. Hemagglutination activity (HA) is defined as the minimum concentration of bean powder (μg/mL) required to agglutinate 50% of the RBC (Bender, 1983).

Hemagglutination activity of bean extracts varied widely among bean types and among cultivars tested. Lowest HA values (indicating strongest agglutination reaction) were found for Aurora (SW), Sanilac and Hyden (N), Roza (PK), Rufus (RM), and both Red Kidney cultivars; these bean extracts agglutinated all four types of R BC tested (Type A; Jaffe, 1972). Lectins in Chief (SW), Harris and UI-59 (GN), Sutter and Viva (PK), Black Turtle Soup (B), and Fiesta (P) agglutinated bovine RBC less strongly than rabbit, rat or human 0^+. Very little or no agglutination resulted at the concentrations tested from GN-1140, pintos Holberg, Nodak, Olathe, Pindak, JM-126, NW-410, NW-590, and UI-114, and the snap bean Blue Mountain (Jaffe's Type D). No lectins were found which agglutinated only treated bovine RBC (Jaffe's Type C).

Nutritional evaluation

Results of the Net Protein Utilization (NPU) trials are given in Table 3. Negative NPU values resulted when the source of protein in rat diets was Aurora, Sanilac, Roza, Rufus, Red Kloud, or Royal Red beans. NPU values <10 occurred from feeding Chief, Viva, or Black Turtle Soup beans. JM-126, Olathe, Sutter or Fiesta beans gave NPU values between 10 and 15. Values of 16-25 resulted from feeding Hyden, Nodak, UI-59, UI-114, Blue Mountain, NW-590, Harris, and Hol-

Table 2—Hemagglutination activity[a] of extracts of 24 bean (Phaseolus vulgaris) cultivar

Cultivar	Rabbit RBC	Human O RBC	Pronase-treated Rat RBC	Trypsin-treated Bovine RBC
Small White				
Aurora	98[b]	390	3	49
Chief	390	781	24	1563
Navy				
Hyden	195	1563	12	195
Sanilac	98	390	6	781
Great Northern				
Harris	390	781	6	1563
GN-1140	25000	25000	49	25000
UI-59	390	781	12	1563
Pink				
Roza	195	3125	6	98
Sutter	390	781	24	1563
Viva	390	781	12	1563
Red Mexican				
Rufus	195	781	6	781
Pinto				
Fiesta	390	781	12	1563
Holberg	25000	25000	98	6250
Nodak	25000	25000	195	25000
Olathe	25000	25000	49	25000
Pindak	25000	25000	195	25000
JM-126	25000	25000	98	25000
NW-410	25000	25000	98	25000
NW-590	25000	25000	195	25000
UI-114	25000	25000	98	25000
Black				
Black Turtle Soup	390	781	12	781
Red Kidney				
Red Kloud	195	1563	3	49
Royal Red	195	1563	1.5	49
Green Pod				
Blue Mountain	25000	25000	24	25000

[a] Hemagglutination activity (HA) = minimum concentration of bean powder (μg/mL) required to agglutinate 50% of the RBC.
[b] Each value is mean of three replicates. S.D. = 1 dilution or less.

Table 3—Nutritional evaluation of 24 bean cultivars

Cultivar	N in ground beans %	Net protein utilization[a]	Apparent digestibility (*in vivo*)	Digestibility (*in vitro*)
Small White				
Aurora	3.37	NEG[b]	23.	77.
Chief	3.13	8.	30.	78.
Navy				
Hyden	3.38	16.	22.	78.
Sanilac	3.33	NEG	21.	76.
Great Northern				
Harris	3.77	23.	54.	78.
GN-1140	3.18	49.	56.	81.
UI-59	3.62	19.	23.	80.
Pink				
Roza	3.11	NEG	18.	77.
Sutter	3.47	15.	18.	80.
Viva	3.31	9.	23.	78.
Red Mexican				
Rufus	3.49	NEG	16.	76.
Pinto				
Fiesta	3.30	15.	52.	77.
Holberg	3.04	25.	24.	80.
Nodak	3.47	17.	22.	77.
Olathe	3.66	14.	52.	75.
Pindak	2.96	56.	49.	79.
JM-126	3.25	12.	25.	78.
NW-410	3.11	34.	47.	79.
NW-590	2.73	22.	20.	78.
UI-114	3.29	19.	51.	79.
Black				
Black Turtle Soup	3.56	5.	24.	77.
Red Kidney				
Red Kloud	3.32	NEG	16.	77.
Royal Red	3.94	NEG	14.	76.
Snap Bean				
Blue Mountain	3.71	20.	53.	82.
Casein	13.64	63.	88.	87.

[a] 10% protein from bean powder or casein in each test diet.
[b] $\text{NPU} = \frac{\text{body N (g) of test protein group - body N (g) of protein-free group}}{\text{N intake (g) of test group}} \times 100$

berg. NW-410, GN-1140, and Pindak had NPU values >25. The NPU for casein was 63. Other researchers have reported NPU values for casein of 60 (Miller and Bender, 1955) and 73 (Palmer et al, 1973); for "Processor" Snap Beans, negative (Palmer et al., 1973), 7 (Pusztai et al., 1979b), and 11 (Pusztai and Palmer, 1977); for GN-1140, 37 and UI-111, 44 (Pusztai et al., 1979b).

Gain or loss in weight of each group of rats (fed either a test diet, casein diet, or nonprotein diet) is shown in Table 4. All rats fed the casein diet gained weight, for a mean gain of 98.7g per group. Rats fed the nonprotein diet lost (mean) 82.3g per group. Rats which lost more weight than their nonprotein controls were fed Aurora, Sanilac, Roza, Rufus, Black Turtle Soup, Royal Red or Red Kloud beans. Weight losses similar to those of the nonprotein controls resulted from feeding Chief, Hyden, Sutter, Viva, or Fiesta beans. The other 12 groups of rats lost less weight (23–53g per group).

Digestibility determinations

In vitro digestibility values and apparent digestibilities determined *in vivo* during the rat-feeding study are given in Table 3. *In vivo* digestibility values reported by other researchers include: casein, 93 and kidney bean, 55 (Palmer et al., 1973); white snap bean, 65, Pinto UI-111, 64 and GN-1140, 71 (Pusztai et al., 1979b); French bean Lingot Blanc, 82 (Rouanet et al., 1985). Hsu et al. (1977) reported an *in vitro* digestibility value for casein of 89 and for bean protein concentrate of 84.

Electrophoresis studies

SDS-gel electrophoresis patterns of extracts of all 24 cvs. (raw) showed strong lectin bands in Aurora, Sanilac, Roza, Rufus, Royal Red, Red Kloud, Black Turtle Soup, Chief, Viva and Fiesta. Visible bands were apparent in Hyden, Sutter, and Harris. These bands represent proteins of approximately 31,000 daltons mol. wt. according to measurements taken from standards used (pepsin, mol. wt. 34,700 daltons; trypsinogen, 24,000 daltons; Fig. 1). Andrews (1974) isolated from beans a lectin component of mol. wt. 114,000 daltons composed of four subunits of mol. wt. approximately 30,000 daltons each.

DISCUSSION

TOXICITY of lectins in raw beans was reported by Liener (1979b) whose co-workers fed purified preparations of black or kidney bean lectins to rats, causing impaired growth or death. Pusztai and Palmer (1977) also found that lectins in kidney bean were responsible for nutritional toxicity (impaired growth and death) to rats; conversely, kidney bean preparations without lectins (lectins removed by affinity chromatography) were non-toxic (no effect on growth; no mortality). Liener (1979b) equated lectins (hemagglutinins) with nutritional toxicity and suggested that testing the hemagglutinating activity of seed extracts against several species of RBC, especially trypsinated bovine RBC, might be a useful system for identifying toxic beans.

A number of researchers have reported antinutritional factors other than lectins in raw beans. Liener (1979a) discussed the deleterious effects of trypsin inhibitor, which causes pancreatic hypertrophy. Bressani et al. (1983) reported that content of condensed tannins (polyphenols) in Guatemalan red beans correlated negatively (non-significant) with net protein ratio (NPR, an estimation of protein quality) but positively (non-signifi-

Table 4—Weight loss or gain[x] of rats fed test diets

Test diet[y]	Weight gain g/group	Weight loss g/group	Slope of[z] wt.-loss curve	Test Diet	Weight loss g/group	Slope of wt.-loss curve
Casein	99.			*Pinto*		
Non-Protein		82.[lmno]	−8.71	Fiesta	61.[ghijkl]	−6.2
				Holberg	34.[abcde]	−4.1
Small White				Nodak	33.[abcde]	−3.5
Aurora		105.[opqrs]	−11.0	Olathe	44.[cdefg]	−5.7
Chief		79.[jklmn]	−9.2	Pindak	28.[abcd]	−3.9
				JM-126	50.[efg]	−5.5
				NW-410	40.[abcde]	−4.8
Navy				NW-590	32.[abcd]	−3.2
Hyden		71.[ikjlmn]	−6.2	UI-114	42.[bcdef]	−5.9
Sanilac		117.[s]	−7.1			
Great Northern				*Black*		
Harris		44.[defg]	−4.9	Black Turtle Soup	94.[nop]	−9.4
GN-1140		30.[abcd]	−3.9			
UI-59		53.[fg]	−5.8			
Pink				*Red Kidney*		
Roza		106.[pqrs]	−8.1	Red Kloud	112.[rs]	−9.8
Sutter		77.[lmn]	−7.4	Royal Red	111.[qrs]	−10.6
Viva		71.[hijklmn]	−6.8			
Red Mexican				*Snap Bean*		
Rufus		84.[no]	−8.6	Blue Mountain	23.[a]	−3.0

[x] Means of duplicate experiments.

[y] Cornstarch (50%), potato starch (10%), dextrose (15%), corn oil (15%), minerals (5%) and vitamins (5%) plus protein (10%) from bean powder or casein at the expense of the starch.

[z] Linear regression was used to calculate best-fit lines from daily weight records for each test-diet group of rats.

[a-s] Means not followed by the same letter are significantly different at the 5% level according to Analysis of Variance and Duncan's Multiple Range Test.

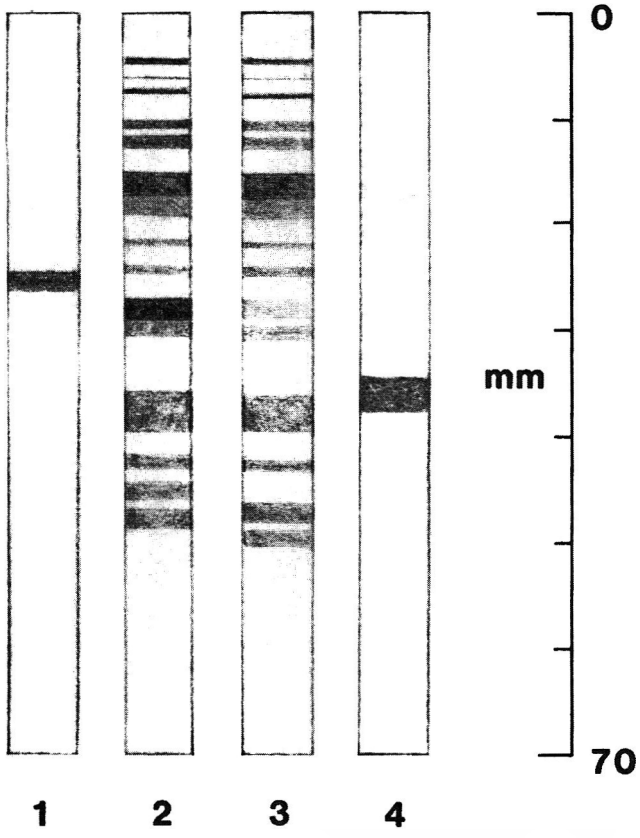

Fig. 1—Graphic representation of SDS-polyacrylamide gel electrophoresis bands for (1) pepsin, 34,700 daltons; (2) Aurora bean powder extract; (3) UI-114 bean powder extract; and (4) trypsinogen, 24,000 daltons M.W.

cant) with apparent (*in vivo*) protein digestibility. Aw and Swanson (1985) found that *in vitro* digestibility and protein quality (*Tetrahymena*) of black beans were inversely related to tannin content. These factors and others may affect digestibility and protein quality as estimated by NPU, NPR, PER (protein efficiency ratio) or *Tetrahymena*.

In vivo digestibility values of the test beans may be low because lectins present interfere with absorption of nutrients through the intestinal mucosa by destroying the epithelial cells in the brush border (Pusztai et al., 1979a).

In vitro digestibility values are based on reactions of bean powder with three peptidases, and do not reflect absorption by animal organisms. Therefore, although *in vivo* and *in vitro* digestibility values are interesting, they seem to be less valid as criteria of lectin toxicity.

On the basis of hemagglutination tests, nutritional studies, and electrophoresis patterns obtained in these experiments, the 24 cvs. tested can be listed according to their lectin toxicity (Table 5).

Brown et al. (1982a) analyzed single seeds of 107 bean cvs., including eight cvs. reported in this study. They identified Sanilac, Rufus, Viva, Sutter Pink and Black Turtle Soup as toxic cvs. with high agglutinating ability and strong lectin electrophoresis patterns, but reported Pintos UI-111 and UI-114 and Great Norterns GN-1140 and UI-59 to be non-agglutinating with no lectin electrophoresis bands.

The fact that Pinto bean cvs., except for Fiesta, were low in lectin content suggests an interesting genetic relationship. Low-lectin Pinto cvs. UI-114 and UI-111 (Pusztai et al., 1981b) were released by the University of Idaho and are closely related, one or the other appearing in the ancestry of most of the other pintos. Because the genetic history of the proprietary cv. Fiesta is not available, its elevated inheritance of lectin content cannot be addressed.

Content of toxic lectins in raw beans may be relatively unimportant in North America and Europe, except for slow or crock-pot cookery. But lectins could be a concern in areas of the world where beans are consumed without being cooked adequately to inactivate the toxicity, because fuel is scarce or water boils at temperatures less than 90°C (Korte, 1972). In these areas, cultivars with low lectin content are potentially more healthful food products than cultivars exhibiting a high lectin content with high-to-moderate toxicity.

One solution to the lectin problem may be to breed new cvs. having negligible levels of toxic lectins (Korte, 1972; Osborn and Bliss, 1985). The toxic lectin characteristic is genetically controlled and inherited as a single dominant gene (Jaffe et al., 1972; Brown et al., 1981a), and experiments to develop

Table 5—Lectin toxicity of 24 cultivars tested

Toxicity	Very toxic	Toxic	Nontoxic
Criteria	Strongly agglutinate RBC; Negative NPU values; Strong lectin electrophoresis bands	Agglutinate RBC; NPU values < 20; Visible lectin electrophoresis bands	Do not agglutinate RBC; NPU values > 20; No lectin electrophoresis bands
Cultivars[a]	Sanilac Red Kloud Aurora Royal Red Roza Rufus Black Turtle Soup	Chief Sutter Viva Fiesta Hyden UI-59 Harris	JM-126 Olathe UI-114 NW-410 Holberg Nodak Pindak NW-590 GN-1140 Blue Mountain

[a] In descending order of toxicity.

lectin-free or low-lectin cultivars are underway at several plant genetics laboratories (Osborn and Bliss, 1985; Silbernagel, 1984). Osborn and Bliss (1985) point out, however, that lectins may be involved in important plant host:pathogen relationships.

Results of this study show that complicated procedures are not necessary for identification of bean lectin toxicity, and that a combination of hemagglutination tests and SDS-polyacrylamide gel electophoresis is suitable for rapid, inexpensive screening of dry bean cvs. for toxic lectins.

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Effects of Microwave Heating on the Molecular Species of Soybean Triacylglycerols

HIROMI YOSHIDA and GORO KAJIMOTO

ABSTRACT

Microwave heating of whole soybeans resulted in an apparent increase of the total lipids. Molecular species of triacylglycerols were isolated from total lipids by a combination of silicic acid column chromatography and $AgNO_3$-thin layer chromatography (TLC). Fifteen molecular species of triacylglycerols were still found in whole soybeans following heat treatment. Microwave heating for about 5 min did not change all molecular species of soybean triacylglycerols nor cause a loss of unsaturated fatty acids. However, heat treatment for 8 min caused a significant decrease not only in molecular species containing more than four double bonds, but also in the amount of diene and triene present in a triacylglycerol.

INTRODUCTION

A HEATING PROCESS to remove antinutritional factors is essential in the use of soybeans as a food or a feed (Rackis, 1974). Improved growth and milk production of animals fed soybeans occurred when the soybeans were heated (Hill and Harshburger, 1979; Mielke and Schingoethe, 1980). Various heating methods have been used to improve the nutritional value of soybeans. Investigations showed that this is due partially to a change in trypsin inhibitor and lipoxygenase activities (White et al., 1967; Pour-El and Peck, 1973; Coliins and Beaty, 1980; Pour-El et al., 1981; Hafez et al., 1984). Microwave heating is one of the processes that has been used to investigate nutritional factors in foods. Some disadvantages of microwave heating include inadequate browning, lack of texture development, "crisping" in certain products, and uneven heating. On the other hand, advantages are convenience, speed, and reduced nutrient loss as a result of elution or chemical alteration. Thus, compared to conventional methods, microwave heating resulted in greater retention of ascorbic acid in fruits and vegetables (Thermador, 1978), and also less alternation of fatty acid composition in beans (Hafez et al., 1985). However, many studies have been conducted on the fatty acid level of total lipids in soybeans, but triacylglycerol, the main component of the beans, has not been investigated at the molecular level. This study was conducted to determine the effects of microwave treatments on the molecular species composition and fatty acid distribution of soybean triacylglycerols.

MATERIALS & METHODS

Soybeans

Commercial soybeans [Glycine Max (L) "Okuhara,"] harvested at Hokkaido Prefecture, Japan, 1984 were selected for uniformity based on bean weight (i.e. between 270.0 to 319.0 mg). The beans were cleaned and divided into nine groups for experimental treatment (0–8 min). The composition of the beans was as follows; Moisture-8.5%, protein-38.6%, fat-18.3%.

Authors Yoshida and Kajimoto are affiliated with the Dept. of Nutrition, Kobe-Gakuin Univ., Arise, Ikawadani-cho, Nishi-ku, Kobe 673, Japan.

Microwave heating

Whole soybeans were heated in the microwave oven for 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 min. The temperature during microwave treatment were recorded from the digital display window of the oven as a function of time, these being 30°, 60°, 90°, 120°, 150°, 160°, 165° and 170°C, respectively. A modified domestic size Sharp microwave (Model R-5550) was used. The oven was rated at 500 watts (full power) at 2450 MHz and was operated at full power. To obtain uniform heating, a turntable was installed which slowly rotated the sample in a direction opposite to the built-in microwave stirrer. We placed whole soybeans in 8.0-cm diameter by 1.5-cm petri dishes such that they completely filled the volume of the dishes with the lids in place. Each petri dish contained ca. 24.8g (85 beans) soybeans, and five dishes were treated once at each of the different exposures to provide sufficient sample material for analysis and testing. After heating, the soybeans were cooled for 20 min before lipid extraction.

Lipid extraction

Microwave treated sample (100 beans) were ground with chloroform-methanol (1:1) at 0°C in a Waring Blendor, and the lipids were extracted 3 times with chloroform-methanol (once at 1:1 and then twice at 2:1) in the Blendor. The combined extracts of total lipids were purified as described earlier (Folch et al., 1957), and then dried over anhydrous Na_2SO_4. The solvents were removed from filtrates with a rotary evaporator under a stream of nitrogen gas at temperatures which did not exceed 35°C. The extracted lipids were stored under nitrogen and frozen at −25°C for further analysis. Samples of raw soybeans were treated in the same manner.

Lipid class analysis

Total lipid extractions were fractionated on a silicic acid column by a modification of a previous method (Rouser et al., 1967). The silicic acid (Unisil 100-200 mesh, Clarkson Co., In., Williamport, PA) was activated at 120°C overnight and again for 1 hr immediately before the column was prepared. All column chromatographic conditions were the same as those previously described (Yoshida and Kajimoto, 1977). After the column was washed with 300 mL chloroform, a measured quantity (200 mg) of total lipids was added with 5 mL chloroform. By successive elution with 200 mL chloroform (fraction I), 700 mL acetone (fraction II) and then 200 mL methanol (fraction III), total lipids were separated into 3 fractions. TLC was used to verify the separations, and when needed, further purification was done with a silica gel column. The nonpolar lipids were eluted in fraction I, the glycolipids containing browning substances in fraction II, and the phospholipids in fraction III. Total concentrations of separated lipid fractions were determined gravimetrically.

Quantitative analysis of nonpolar lipids

The nonpolar lipid fraction eluted from column chromatography was further separated by one-dimensional TLC on Silica Gel G plates activated at 120°C for 3 hr immediately before use. The solvent system was petroleum ether-diethyl ether-acetic acid (80:30:1 or 90:10:1) and *n*-hexane-benzene (1:1). Following development, the plate was exposed to iodine vapor to visualize the ester-type lipid, free fatty acid and sterol bands, which were outlined and scraped into test tubes. After each identified spot was extracted with chloroform, the ester bond was determined by the method of Synder and Stephens (1959). The free fatty acids were methylated with 14% boron trifluoride methanol (Morrison and Smith, 1964), and then estimated by ester bond determination. The calibration curve was determined with methyl palmitate.

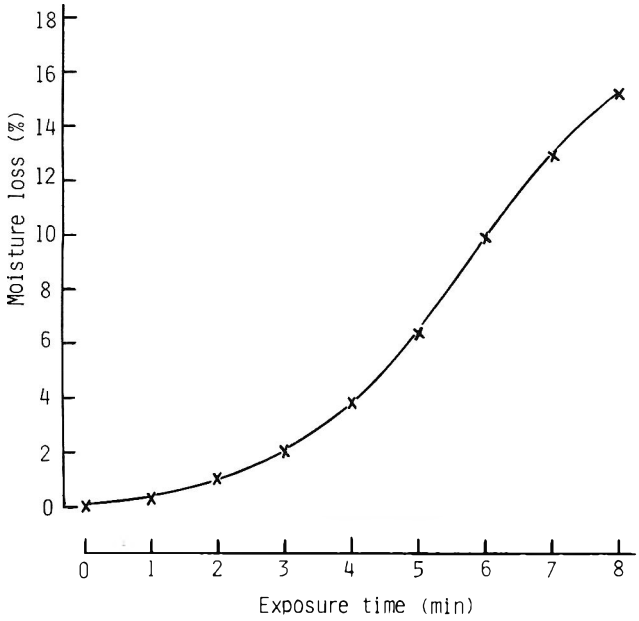

Fig. 1—Relationships between moisture loss and exposure-time during microwave heating at frequency of 2450 MHz.

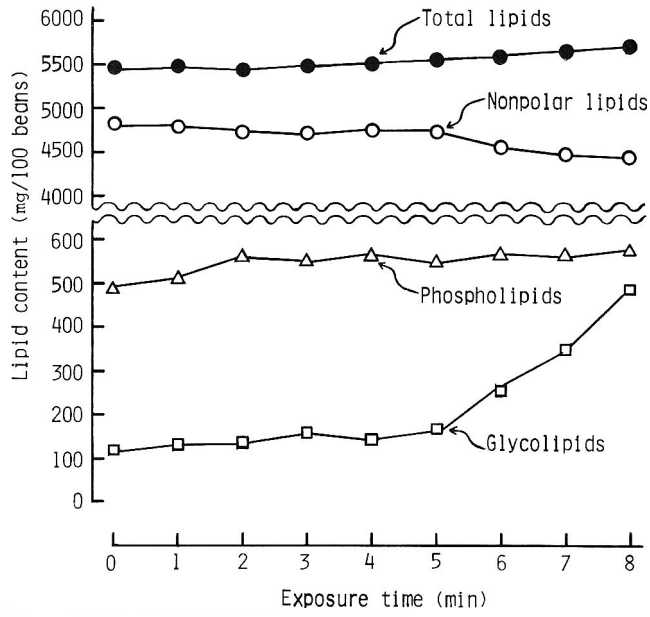

Fig. 2—Changes in lipid contents of soybeans during microwave heating at frequency of 2450 MHz.

Triacylglycerol species analysis

Molecular species analysis of total triacylglycerols was performed by silver nitrate-silica gel TLC (De la Roche et al., 1971). Plates were coated to 0.3 mm thickness with a slurry of 35 g Silica Gel G and 8.8g silver nitrate dissolved in 70 mL water. Freshly prepared plates were activated at 100–120°C for 2 hr, then stored before use in a desiccator in the dark. Triacylglycerol molecular species were separated by argentation TLC using 0.8% to 5.0% methanol in chloroform, depending on their degree of unsaturation (Blank et al., 1965). For quantitation of species containing the trienoic acid (linolenic acid) plates were streaked with 10–15 mg triacylglycerol and developed with 5.0% methanol in chloroform. Remaining species were separated by streaking 8–10 mg triacylglycerol on the plates and developing with 0.8–1.5% methanol in chloroform. This system was varied according to temperature and humidity conditions. Individual bands were visualized under ultraviolet light after spraying with 0.2% 2′, 7′-dichlorofluorescein in ethanol. Bands were recovered from the plate by extraction with 10% methanol in diethyl ether in a Buchner funnel, followed by acidification of the adsorbent with 10% aqueous HCl in a separatory funnel and extraction with diethyl ether. Determination of relative amounts of each triacylglycerol subfraction was carried out by comparison of fatty acid methyl esters with a known amount (20 μg) of methyl pentadecanoate as an internal standard. The subfraction was converted into fatty acid methyl esters by heating it with 14% boron trifluoride methanol (Morrison and Smith, 1964), and analyzed by gas chromatography (GC) as described earlier (Yoshida and Kajimoto, 1977). Peak areas were calibrated with standard fatty acid mixtures (F & OR mixtures No. 3, Applied Science, State College, PA), and calculated as mole% of each fatty acid with the aid of an electronic integrator (Shimadzu C-RIB). The other GC conditions were the same as previously described (Yoshida and Kajimoto, 1980).

Statistical analyses were conducted with the Student's t-test (Steel and Torrie, 1960).

RESULTS & DISCUSSION

THE EFFECTS of microwave heating of whole soybeans on moisture loss is shown in Fig. 1. The loss depended upon total volatile substances, but it was considered to be mostly moisture. Small reductions were observed in the initial stage of heating, by 1.0% within 2 min and 4.0% by 4 min. After 4 min of heating, however, there was a linear relationship between moisture loss and exposure-time. At the 8 min of heating the loss was ca. 15% of the weight of whole soybeans before heating, and the beans lost texture (crisping). Therefore, optimum microwave heating time was around 5 min for whole soybeans to prepare soyflour or soy grits under the experimental conditions in this study.

Changes in the amounts of lipid components in the whole soybeans during microwave heating are shown in Fig. 2. Total lipids increased during the microwave heating process, in agreement with the finding of Collins and Beaty (1980) that heat caused a certain amount of protein denaturation which could have improved lipid extractability. Nonpolar lipids, which were the major lipid component in the soybeans, showed a significant decrease after 5 min of heating. The loss was ca. 350 mg per 100 beans between 5 and 8 min. In contrast, as the nonpolar lipid concentration gradually decreased, the glycolipid component increased considerably, and the phospholipid component also increased slightly. Hafez et al. (1984) reported that an increase of browning substances was observed when soybean seeds were heated by using a microwave oven. The browning substances are generally very polar due to several active radicals in the compounds (Gomyo and Horikoshi, 1976; Kawakishi et al., 1983). This may be attributed to the increase of glycolipids because their sugar moiety could produce browning substances. We also might expect such differences because lipid extraction was carried out after heat treatment rather than before heat treatment as in the conventional process. Tomioka and Kaneda (1974) reported that browning products formed in heated lecithin seemed to be compounds retaining the structure of original lecithin, but polymerized at the fatty acid parts.

The changes in the amounts of nonpolar lipid components during microwave heating are shown in Fig. 3. The dominant components were triacylglycerols, with much smaller amounts of monoacylglycerols, 1,3-diacylglycerols, free fatty acids and 1,2-diacylglycerols. A distinct difference in the amounts of individual lipids was observed after 5 min when there was a considerable linear loss in the vaporization of water. The amount of triacylglycerols gradually decreased by 3.0% within 5 min and 17.0% in 8 min. Conversely, most minor components rapidly increased by a range from 3-fold for free fatty acids to 6-fold for 1,2-diacylglycerols within 8 min heating. On the other hand, the 1,3-diacylglycerols peaked at 5 min of heating, and decreased thereafter. The results indicated that the triacylglycerol hydrolysis in soybeans during microwave heating involved energy absorption related to the temperature and vaporization of water (Pour-El et al., 1981). Although some of the water vaporized during the microwave heating process, and

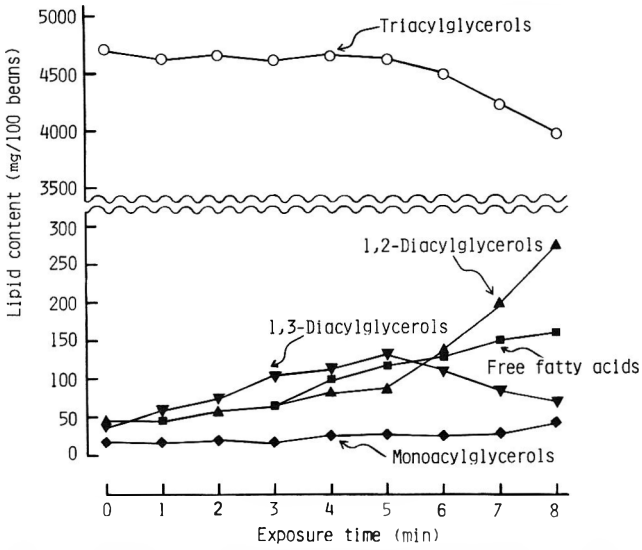

Fig. 3—Changes in nonpolar lipid contents of soybeans during microwave heating at frequency of 2450 MHz.

Table 1—Residual molecular species of triacylglycerols isolated from soybeans before and after microwave heating[a,b]

Triacylglycerol species[c]	No. of double bonds	% total at min 0	Exposure time (min) 0	5	8
S_2M	1	1.9	88.9[d]	87.5[d]	86.7[d]
SM_2	2	3.1	145.1[d]	145.3[d]	143.8[d]
S_2D	2	3.8	177.9[d]	175.6[d]	173.0[d]
M_3	3	2.4	112.3[d]	120.5[d]	115.6[d]
SMD	3	8.5	397.8[d]	392.8[d]	387.3[d]
M_2D	4	6.9	322.9[d]	314.7[d,e]	290.7[e]
SD_2	4	13.7	641.2[d]	634.8[d,e]	582.4[e]
SMT	4	3.2	149.8[d]	143.3[d]	112.4[e]
MD_2	5	13.5	631.9[d]	628.2[d]	542.8[e]
M_2T	5	2.3	107.7[d]	101.8[d]	87.6[e]
SDT	5	3.6	168.5[d]	160.3[d]	138.7[e]
D_3	6	18.5	865.9[c]	858.7[d]	703.5[e]
MDT	6	2.8	131.1[d]	130.3[d]	100.8[e]
D_2T	7	11.5	538.3[d]	526.1[d]	374.1[f]
DT_2	8	4.3	201.3[d]	191.3[d]	105.3[f]

[a] Each value is an average of 3 determinations.

[b] The residual amounts were calculated in comparision with a known amount methyl pentadecanoate as an internal standard and expressed as mg triacylglycerol per 100 beans.

[c] Abbreviations: S, saturated; M, monoenoic; D, dienoic; T, trienoic acid moieties of triacylglycerols, e.g., SMD, a triacylglycerol containing one saturated, one monoenoic and one dienoic fatty acid; D_3, trilinolein.

[d,e,f] Values in a row with different superscripts are significantly different from those at min 0 ($d_p < 0.05$, $e_p < 0.02$, $f_p < 0.01$).

condensed and was reabsorbed by the soybeans as they cooled in the petri dishes, the net moisture loss was substantial (Fig. 1).

Table 1 illustrates the residual molecular species of triacylglycerols isolated from soybeans before and after microwave heating. Saturated fatty acids are denoted as S and include palmitic (16:0) and stearic (18:0) acids. Unsaturated fatty acids, oleic (18:1), linoleic (18:2) and linolenic (18:3), are designated as monoene (M), diene (D) and triene (T), respectively. In all, fifteen different molecular species were detected by TLC. but four of these containing monoene (S_2M, M_3, M_2T and MDT) were very small components (below 3.0%). Conversely, four species containing diene (SD_2, MD_2, D_3 and D_2T) were above 11.0%, and the major components of soybean triacylglycerols. The other seven species (SM_2, S_2D, SMD, M_2D, SMT, SDT and DT_2) ranged from 3.1% to 8.5%. There was no distinct significant change in the amounts of triacylglycerol molecular species with 5 min of microwave heating. However, heating for 8 min caused a significant change in the molecular species containing more than 4 double bonds; and with few exceptions (SMT and SDT), the higher the unsaturation, the greater the loss of the species (10% for 4 double bonds, 14–27% for 5–6 double bonds and 30–48% for 7–8 double bonds, respectively).

Table 2—Content of fatty acids in the triacylglycerols isolated from soybeans before and after microwave heating[a]

	Fatty acid[b]	Exposure time (min) 0	5	8
Found[c]	S	669.3[e] (100)[h]	660.9[e] (98.7)	639.0[e] (95.5)
	M	964.2[e] (100)	908.9[e,f] (94.3)	883.3[f] (91.6)
	D	2541.6[e] (100)	2522.3[e] (98.5)	2114.3[f] (83.2)
	T	505.5[e] (100)	498.0[e] (98.5)	347.1[g] (68.6)
Calculated[d]	S	678.7[e] (100)	667.6[e] (98.4)	627.9[e] (92.5)
	M	962.5[e] (100)	955.8[e] (99.3)	873.7[e] (92.5)
	D	2540.1[e] (100)	2506.3[e] (98.7)	2101.7[f] (82.7)
	T	499.3[e] (100)	481.4[e] (96.4)	341.4[g] (68.4)

[a] Each value is an average of 3 determinations and expressed as mg fatty acid per 100 beans.

[b] Abbreviations: see Table 1.

[c] Values obtained by GC in comparison with a known amount of methyl pentadecanoate as an internal standard using triacylglycerols isolated from soybeans.

[d] Calculated from the relative percentages of each triacylglycerol species based on data in Table 1.

[e,f,g] Values in a row with different superscripts are significantly different from those at min 0 ($e_p < 0.05$, $f_p < 0.02$, $g_p < 0.01$).

[h] Values in parentheses are for remaining fatty acid (%).

Table 2 shows the content of fatty acids in the triacylglycerols isolated from raw and microwave treated soybeans, expressed as mg per 100 beans according to their degree of unsaturation. The theoretical contents of fatty acids were calculated from the relative percentages of each triacylglycerol species based on the data in Table 1 and their distribution of each fatty acid which composed of the individual molecular species. These values are compared with the experimental values (Table 2). There were no quantitative or qualitative differences in the distribution between the found and calculated (theoretical) values. Mai et al. (1980) showed that the fatty acid composition of peanuts remained unchanged after microwave cooking up to 15 min. However, we observed that microwave heating for 8 min caused decreases from ca. 430 mg (17%) for diene to ca. 160 mg (30%) for triene. In conclusion, contrary to the reports of Mai et al. (1980) and Hafez et al. (1985) using peanuts and soybeans, respectively, there were significant decreases not only in the several molecular species containing more than 4 double bonds, but also in the amount of diene and triene of triacylglycerols when soybeans were microwave treated for 8 min. It should be pointed out that microwaving time would vary according to the weight of the sample, and the power of the oven.

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Disulfide Reduction and Molecular Dissociation Improves the Proteolysis of Soy Glycinin by Pancreatin *in vitro*

E. ROTHENBUHLER and J. E. KINSELLA

ABSTRACT

THE EFFECTS of structural modification by urea and dithiothreitol on the *in vitro* pancreatin proteolysis of soy glycinin was studied. Urea, up to 4.5M, which causes dissociation of glycinin into subunits and some unfolding of the polypeptides progressively increased proteolysis by pancreatin as measured by the pH-stat method. Reduction of the intermolecular disulfide bonds with dithiothreitol doubled the rate of proteolysis and when additional intramolecular disulfide bonds of glycinin were cleaved, the rate of digestibility increased approximately threefold becoming equivalent to casein in its susceptibility to proteolysis by pancreatin.

INTRODUCTION

A NUMBER of factors, such as trypsin inhibitors, may limit the digestibility of soy globulins and other legume proteins (Kakade and Liener, 1974; Wilcke et al., 1979; Liener, 1979). However, Liener reported that when the antinutritional factors were removed, the full biological value of soy proteins was not realized, i.e, only about 80% of the theoretical biological value was observed. It is conceivable that the inherent structural features of the globular storage proteins of legumes may render them relatively more resistant to some digestive enzymes than other less structured food proteins such as casein (Fukushima, 1968; Boonvisut and Whitaker, 1976; Rothenbuhler and Kinsella, 1985). In vivo, soy protein isolate is quite digestible and can be an adequate source of protein for humans (Scrimshaw et al., 1983).

To test the possibility that the quaternary and tertiary structure of globulins (particularly the tightly folded disulfide linked components of the glycinin) impart resistance to proteolytic hydrolysis it should be informative to study the effects of structural modification of pure glycinin (11S) on its susceptibility to proteases. Treatment of 11S with urea causes dissociation of the 320,000 dalton oligomer into two half molecules of 160,000 daltons each which further dissociate into disulfide-linked subunits (Lillford, 1978; Kinsella et al., 1985). Treatment with dithiothreitol by reducing the intermolecular disulfide bonds results in dissociation of the acidic and basic polypeptides and depending on concentration of DTT, reduction of some intramolecular disulfide bonds may also occur (Kinsella et al., 1985; Kella et al., 1986). The objective of this study was to assess the effects of structural modification on the susceptibility of soy 11S to proteolysis particularly the possible relationship between rates of hydrolysis and the number of disulfide bonds reduced. Pancreatin (trypsin and chymotrypsin) which is sensitive to the conformation of protein substrates was used to monitor the effects of molecular alteration on digestibility because pepsin requires acidic conditions which can alter the structure of 11S (Lynch et al., 1977a).

MATERIALS & METHODS

GLYCININ (soy 11S protein), free of trypsin inhibitors, was isolated from defatted, minimally treated soy flour (Central Soya, Fort Wayne, IN). Pancreatin, (porcine pancreatin, grade 3) and sodium caseinate was purchased from Sigma Chemical Co. (St. Louis, MO). Analytical grade chemicals and double distilled water were used in all the experiments.

Authors Rothenbuhler and Kinsella are with the Institute of Food Science, Cornell Univ., Ithaca, NY 14850.

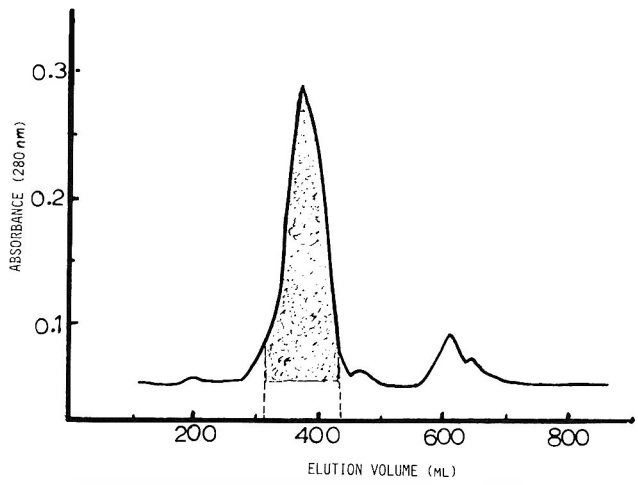

Fig. 1—Elution profile of glycinin from Sepharose-6B. The major peak is glycinin which was homogeneous by SDS-PAGE electrophoresis.

Preparation of 11S

Soy proteins were extracted from the low heat flour with Tris-HCl buffer 0.03 M containing 10mM mercaptoethanol (MeSH) at pH 8.0. The crude 11S was isolated by the procedure of Thanh and Shibasaki (1978) obtaining approximately 10g of crude 11S from 50g of soy meal. The 11S was further purified using Sepharose-6B gel chromotography in a glass column containing 750 mL bed volume of gel. The column was equilibrated with 0.035M potassium phosphate buffer, pH 7.6, containing 0.4M NaCl and 1 μM (MeSH). The column was loaded with 750 mg protein in 10 mL of phosphate buffer which was eluted at the rate of 1 mL per minute. Fractions (1 μM) were collected and protein was monitored by absorption at 280 nm. Fractions containing 11S were pooled and freeze-dried. The yield was approximately 550 mg or 73% of the applied protein.

Electrophoresis

Protein subunits, acidic and basic polypeptides, were separated electrophoretically under denaturing and reducing conditions (Laemmli, 1970; Utsumi and Kinsella, 1985). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out using separating slab gels consisting of linear gradients of 5 – 20% acrylamide. Samples of protein (40 μg) were made up in 50 μL of 63 mM Tris HCl dissociating buffer, pH 6.8, containing 8M urea, 1% SDS, 0.5M dithiothreitol DTT, and 0.025% bromphenol blue. Some samples were run under nondissociating conditions, i.e, without pretreatment with urea, SDS or DTT.

Reduction of disulfide bonds

To determine the effects of the dissociation of the quaternary intermediate subunits of 11S into individual acidic and basic polypeptides and the effects of reduction of intramolecular disulfide bonds of these subunits on digestibility, the 11S protein was progressively reduced with DTT. Pure 11S (50 mg) was dissolved in 50 mL of nitrogen saturated 0.03M Tris-HCl buffer pH 8 (containing 5M urea 1mM

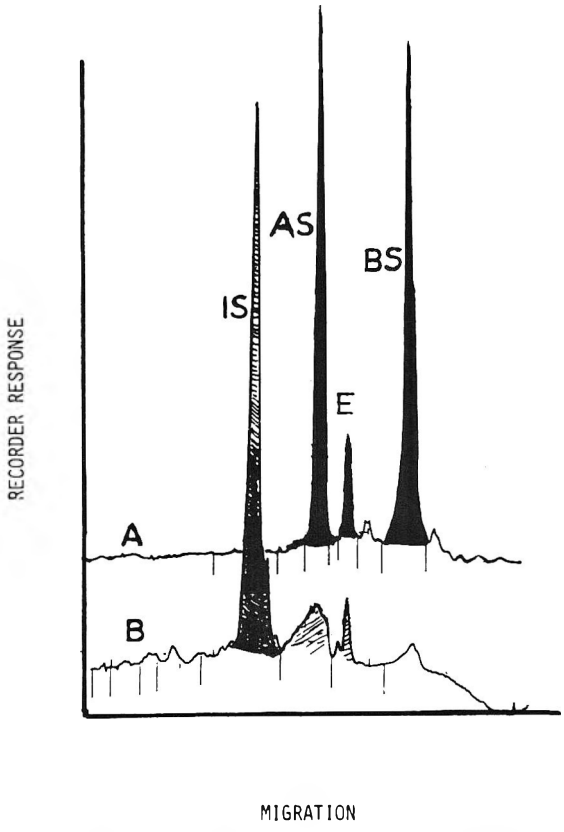

Fig. 2—Scan of electrophoretogram of pure glycinin A dissociated with urea 8M and reduced with dithiothreitol 0.5M to yield acidic (AS), basic (BS), and extra acidic (E) polypeptides and *B* glycinin dissociated into its dimeric disulfide-linked subunits (1S) with 8M urea.

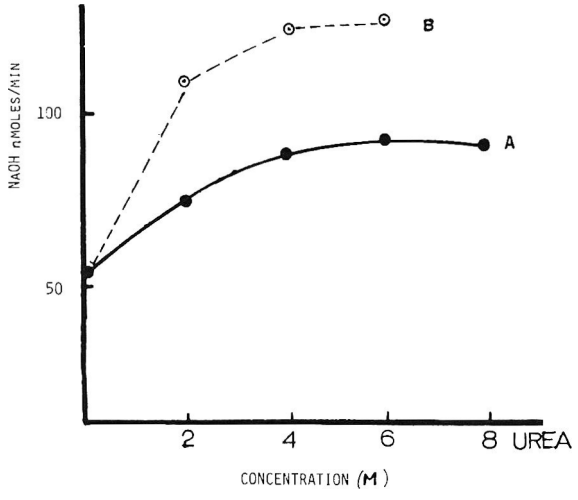

Fig. 3—Effects of increasing urea concentration (0–8M) (A) and urea (M) plus 10 mM dithiothreitol (*B*) on pancreatin hydrolysis of soy glycinin.

EDTA and 0.02% sodium azide) and DTT was added at 0.1, 0.5, 1.0, 2.0, and 10 mM (Fig. 4). Solutions were saturated with nitrogen, and the mixture was incubated at 37°C for 6 hr., dialyzed against water (pH 8) at 4°C for 48 hr to remove all reagents, and freeze-dried. In addition, samples of 11S were reduced with 10 mM DTT, in the presence of 0, 2, 4, and 6M urea (Fig. 3) and with DTT (1mM) in the presence of 3 and 5M urea (Fig. 6). These samples were dialyzed and freeze-dried.

The concentration of sulfhydryl was determined using the Ellman reagent as described by Janatova et al. (1968). Protein samples containing thiol (SH) groups in the concentration range 25 – 100 μM

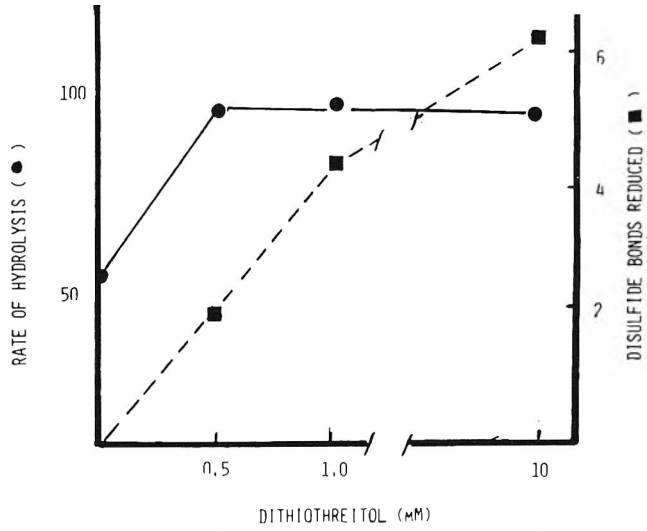

Fig. 4—Effects of dithiothreitol (0–10 mM) pretreatment on the rate of pancreatin hydrolysis (nmoles NaOH used/min) and number of disulfide bonds reduced in glycinin.

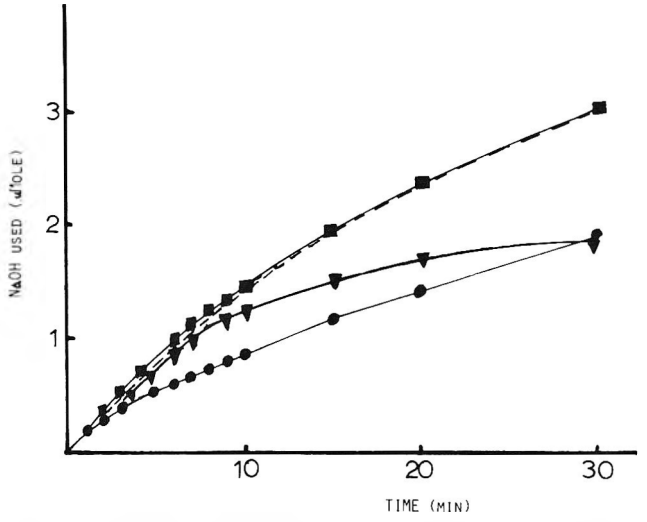

Fig. 5—Relative rates of hydrolysis of glycinin reduced with 1 mM dithiothreitol plus 3 mM urea (■——■); reduced and blocked as the S-Sulfenyl derivatives (■-----■); deblocked reduced glycinin (▼——▼), and unmodified glycinin (●——●). See Methods for details.

were dissolved in 0.8 mL Tris-HCl buffer pH 8 containing 0.2 mL 1% SDS. To this solution, 20 μL 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (10 mM solution in methanol) were added. After 5 min incubation, the absorption at 412 nm was read against the reference (cysteine). Protein was determined by the method of Lowry et al. (1951).

Blocking of thiol groups

In preliminary studies, to determine the possible effects of reoxidation following reduction of 11S, the free SH groups were protected against reoxidation by converting them to s-sulfenyl sulfonate derivatives ($S\text{-}SO_3$) using sodium tetrathionate (Rothenbuhler and Kinsella, 1985). Reduced 11S globulin, (50 mg in 50 mL Tris-HCl buffer 0.03M, pH 7, containing EDTA (1mM) and 0.02% sodium azide NaN_3) was mixed with 2 mL of sodium tetrathionate, 0.23mM for 60 min at 25°C. The material was then dialyzed against water and freeze-dried.

The S-sulfenyl sulfonate substituent groups on 11S were removed using 2-mercaptoethanol. Thus, to 50 mg of the sulfenyl sulfonated 11S in 50 mL Tris-HCl buffer pH 7.6 at 37°C was added 350 μL 0.5mM 2-MeSH and incubated for 15 min at 37°C to regenerate the free SH groups. The material was dialyzed and freeze-dried.

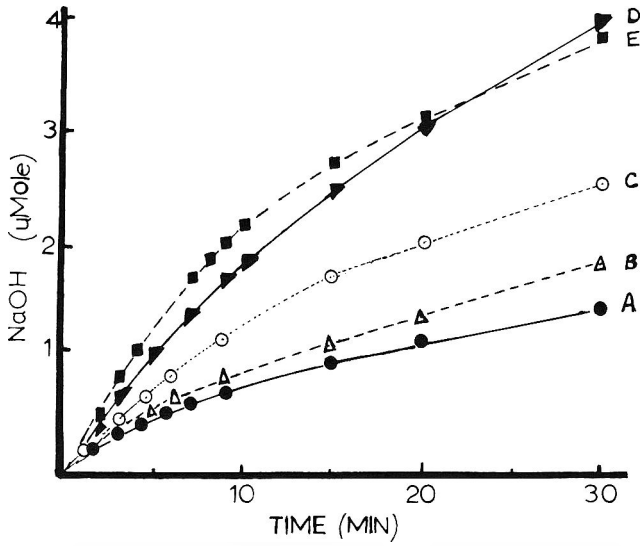

Fig. 6—Effects of reduction by dithiothreitol (1 mM) (in the presence of an increasing concentration of urea) of an increasing number of disulfide bonds on the rates of hydrolysis of glycinin by pancreatin. (A) native glycinin; (B) glycinin reduced with 1 mM DTT alone, (2-4 disulfide bonds reduced); (C) glycinin reduced with 1.0 mM DTT plus 3M urea (4-6 disulfide bonds reduced); (D) glycinin reduced with 1 mM DTT plus 5M urea (> 7 disulfide bonds reduced); and (E) standard sodium caseinate. See Methods for details.

Urea denaturation of 11S

To determine the effects of dissociation and unfolding of the native molecular structure on digestibility, the 11S was treated with urea. Lyophilized 11S (50 mg) was dissolved in 25 mL 0.03M Tris HCl buffer, pH 8, containing 1 mM EDTA and 0.02% sodium azide. This was shaken for 6hr at 37°C with 0, 2, 4, 6, and 8M urea. The solutions were then dialyzed for 48 hr against distilled water pH 8 at 4°C and the product lyophilyzed.

Turbidity measurements

The turbidity of the modified 11S preparations was determined by measuring absorbance at 600 nm of dispersions (1.5 mg/mL) of the protein in Tris-HCl buffer pH 8.

In vitro digestibility

The pH stat method provides a rapid approach for assessing protein digestibility in vitro (Rothenbuhler and Kinsella, 1985; Mihalyi, 1978). The pH stat automatically measures the amount of sodium hydroxide required to neutralize protons liberated during hydrolysis of peptide bonds as discussed in detail by Mihalyi (1978). In this study, we followed the standardized protocol and the procedure for substrate and enzyme preparation as outlined previously (Rothenbuhler and Kinsella, 1985). In a standard run, the protein solution (5 mL) was pipetted into the thermostatted reaction vessel maintained at 37°C. This vessel was continuously flushed with a stream of nitrogen and stirred. The pH was adjusted to 8.0. When the system had equilibrated, 100 μL of pancreatin (1.2 mg/mL) was injected into the vessel and proteolysis was continuously monitored from NaOH consumption using a special microprocessor interface between the burette and the recorder. Linear rates of proteolysis were obtained using substrate concentrations of 3 mg/mL and an enzyme to protein ratio of 1:128 (Rothenbuhler and Kinsella, 1985).

RESULTS & DISCUSSION

CHROMATOGRAPHY on Sepharose-4B colums yielded a single peak for the 11S protein (Fig. 1). Electrophoresis under dissociating conditions showed that this peak gave a single band corresponding to the disulfide linked acidic and basic subunits (Fig. 2). Upon reduction with DTT (0.5M) only acidic and basic polypeptides were obtained (Fig. 2B) indicating the purity of the 11S protein. Incidentally, a small bond corresponding to an extra acidic component was consistently observed on electrophoretograms corroborating the report of Iyengar and Ravestein (1981).

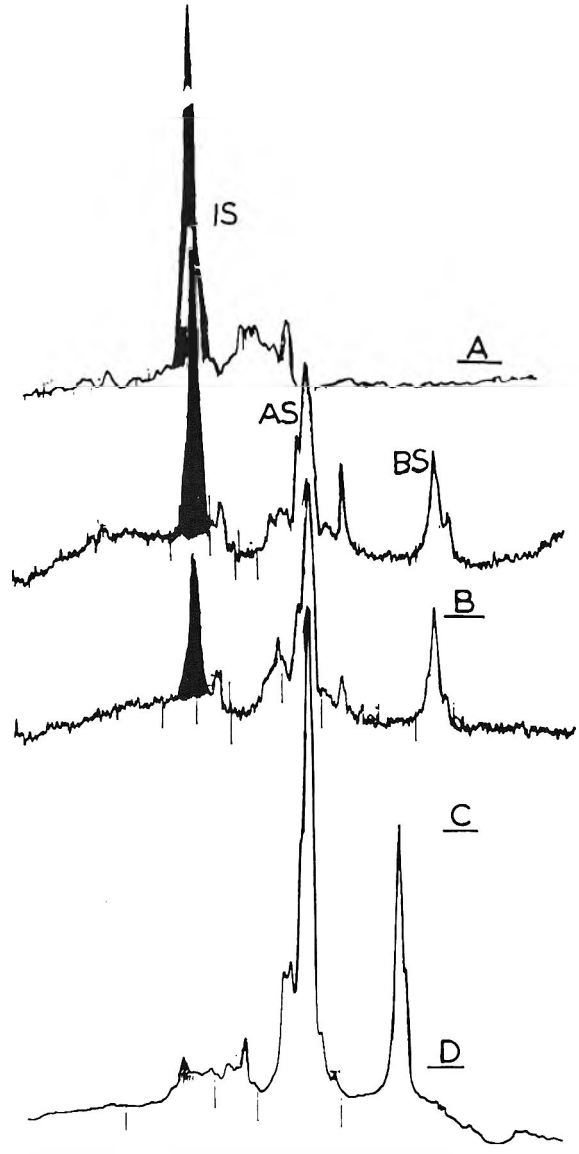

Fig. 7—Scans of SDS-PAGE gels in nondissociating nonreducing buffer of glycinin pretreated with increasing concentrations of dithiothreitol; (A) 0; (B) 0.5; (C) 1.0; and (D) 10 mM DTT, respectively, labeled as in Fig. 2. Scan of A reduced to half original.

Effects of urea

Urea perturbs both hydrophobic interactions and hydrogen bonding association between 11S polypeptide molecules. Since both of these forces are involved in the association of the two half 11S molecules and also of the disulfide linked intermediate acidic/basic dimers (Badley et al., 1975; Kinsella et al., 1985;), the effects of increasing concentrations of urea on digestibility of the 11S was assessed. The solubility, as reflected in absorbance at 600 nm, was only slightly affected by urea up to 8 M, while the viscosity of the 11S progressively increased up to 6M urea after which it remained constant (data not shown). There was a progressive increase in the digestibility of the 11S treated with urea up to 5M (Fig. 3). These data indicated that conformational and structural changes induced by urea facilitated the pancreatin digestibility of soy 11S in vitro.

Lillford (1978) reported that urea up to 1M had limited effects on the integrity of 11S but above 1.5M the 11S began to

dissociate and exposure to increasing concentrations of urea above 4M caused a progressive unfolding of the tertiary structure of 11S. Damodaran and Kinsella (1981) showed that urea concentration up to 4M progressively decreased the fluorescence intensity emission of soy 11S while above 4M the fluorescence intensity increased. These data reflected unfolding of the protein and exposure of tryptophan residues from the interior of the molecule indicating that urea destabilizes hydrophobic interactions. Kamata et al (1979a) reported that trypsin digestibility of 11S was increased approximately threefold with urea treatment up to 4M after which it progressively decreased and at 8 M showed only twice the rate of hydrolysis compared to the native 11S. Kamata et al. (1979b) suggested that some refolding of the 11S occurred during the dialysis step, and this may have accounted for the decrease in digestibility. These workers reported that above 4M urea the beta structure of protein was disrupted but upon dialysis a significant amount of the beta structure was regained, particularly, above 4M urea. In contrast to the data of Kamata et al. (1979a,b), in our studies, 4M urea increased digestibility by only 50%. This may reflect reaggregation of the dissociated 11S components during subsequent dialysis.

Disulfide reduction

The extent to which urea can dissociate and unfold glycinin is limited by the numerous disulfide (S-S) bonds, i.e, the six intermolecular bonds linking the acidic and basic polypeptides and the 11-14 intramolecular S-S linkages in the acidic and basic subunits (Kinsella et al., 1985; Catsimpoolas et al., 1969). Therefore, the effects of reduction of these S-S with DTT in the presence of urea was studied. The inclusion of DTT (0.5 mM) doubled the rate of proteolysis (Fig. 4) reflecting the additional unfolding of the component polypeptides. Subsequent research was conducted to determine the effects of progressive reduction on the changes in 11S and how they affected proteolysis.

The digestibility of 11S by pancreatin after 30 min was almost doubled following reduction of approximately 2-5 disulfide bonds (Fig. 5). Because of the possibility that the reduced thiol groups might be reoxidized during dialysis, in initial experiments these groups were protected by conversion to the S-sulfenyl sulfonate derivatives. This had a negligible effect on the rate of proteolysis (Fig. 5). Following unmasking of the protected thiol groups with mercaptoethanol, the initial rate of proteolysis up to 10 min was comparable to the reduced 11S; however, the subsequent rate of proteolysis progressively decreased up to 30 min. This may indicate that reoxidation of the thiol groups by bubbling air through the sample after mercaptoethanol unmasking caused the formation of polymers which were more resistant to pancreatin. These experiments indicated that protection of the free thiol groups following DTT treatment was unnecessary.

Reduction of more than 4-5 disulfide bonds by DTT (0.5–10mM) alone did not further enhance proteolysis (Fig. 4) presumably because of aggregation of the reduced polypeptides (Damodaran and Kinsella 1982; Kella et al., 1986). Therefore, we examined the effects of urea on reduction. The progressive reduction of disulfide bonds in the presence of increasing concentrations of urea concurrently enhanced the rate of proteolysis by pancreatin (Fig. 6). The reduction of six disulfide bonds, mostly intermolecular disulfide linkages, by treatment with <2 mM DTT doubled the rates of hydrolysis after 30 min. However, when additional disulfide bonds, i.e, 6 intermolecular and some intramolecular disulfide bonds were reduced, the rate of proteolysis was significantly increased and the rate of proteolysis was equivalent to that obtained for casein.

Scans of the electrophoretograms (Fig. 7) indicated that the intermolecular disulfide bonds linking the dimeric intermediate subunits were completely reduced by 1 mM DTT. The peak corresponding to the acidic polypeptide was greater than that of the basic polypeptide particularly following reduction. This conceivably was due to aggregation and precipitation of the dissociated basic subunits following reduction as reported previously (Utsumi and Kinsella, 1985; Damodaran and Kinsella, 1982).

Boonvisut and Whitaker (1976) reported that treatment with β - mercaptoethanol improved the digestibility of soy proteins. Lynch et al. (1977b) showed that cleavage of disulfide bonds of glycinin with β - mercaptoethanol and blockage of the SH groups to yield carboxyamidomethyl glycinin resulted in an enhanced rate of proteolysis. These workers reported that the acidic subunits were apparently more susceptible to proteolysis than the basic components. Recently Kella et al. (1986) confirmed this observation which reflects the slower rate of proteolysis of the precipitated basic polypeptides.

The enhanced rates of digestibility of 11S treated with DTT and urea probably reflects the enhanced solubility of the resultant protein mixture especially the basic components though the DTT and urea were dialyzed before hydrolysis. Recent research indicated that the basic polypeptides seem to be slightly more resistant to pepsin digestion than the acidic subunits; however, they are readily hydrolyzed by both pepsin and pancreatin once they are solubilized (Kella et al., 1986).

The above data support the suggestion that the native tertiary and quaternary structure of soy 11S may impair the susceptibility of soy glycinin to in vitro proteolysis. However, prior acid treatment of soy 11S improves its susceptibility to pancreatic digestibility (Rothenbuhler and Kinsella, 1985) and recent results indicated that sequential peptic and pancreatic proteolysis hydrolyze both the acidic and basic polypeptides of soy 11S though the latter were initially more resistant (Kella et al., 1986). This is consistent with human feeding studies which reveal the high bioavailability of isolated soy protein (Scrimshaw et al., 1983). Research to determine if treatment with disulfide reducing agents can further improve the biological value of soy proteins (in which trypsin inhibitor and other antinutritional factors are inactivated) might be worthwhile as such information should be useful in formulating infant and weaning foods and feeds for young animals using soy proteins.

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Physicochemical Characteristics of 18 Cultivars of Nigerian Cowpeas (*V. uniguiculata*) and Their Cooking Properties

I. O. AKINYELE, A. O. ONIGBINDE, M. A. HUSSAIN, and A. OMOLOLU

ABSTRACT

Eighteen cultivars of cowpeas (*V. unguiculata*) were analyzed for cooking time, swelling capacity, leached solid, percent seed coat, seed density, amylose, tannins, proteins, and soluble sugars. The physicochemical characteristics varied with the seed coat color, the hilum color, and the skin texture. The cooking time was positively correlated to protein, seed density, percent seed coat, swelling capacity and leached solids, and negatively correlated to the levels of amylose, soluble sugars and tannins. There were significant correlations ($p < 0.05$) between sugars and swelling capacity, protein and seed density; and seed density and swelling capacity. The mean percent seed coat was significantly different ($p < 0.05$) between the varieties with wrinkled seed coats and those with smooth seed coats.

INTRODUCTION

COWPEAS are widely consumed in different forms in Nigeria and other West African countries. Various types of products are traditionally produced by cowpeas by soaking, dehulling, grinding, boiling or frying. Legumes constitute the major source of plant protein in Nigerian diets. Studies have been carried out to understand the cooking mechanism in beans. The presence of chelating agents which affect the stability of divalent cations (Rockland and Jone, 1974), the level of phytic acid (Rosenbaum et al; 1965; Longe, 1983), the thickness of the palisade layer (Muller, 1967), the amylose/amylopectin level (Juliano et al; 1965), length of storage (Jackson and Varriano-Marston, 1981) and structural and chemical differences in seed coat components (Hambly, 1932) have been associated with the cooking quality of cereals and legumes. Ojomo and Chheda (1972) reported that cowpea varieties with smooth and wrinkled seed coat have thicker testa than those with rough seed coat which in-turn affected the rate of water absorption.

Cultivars with comparatively thick, smooth seed coats had a slow initial rate of water absorption, whereas thin seed coat cultivars had high initial rate and dehulled better after soaking (Sefa-Dedeh et al; 1979). The choice of cowpeas by Nigerian women is guided predominantly by the cooking time, swelling capacity, taste, and color (Hussain et al; 1984). The aim of this study was to investigate the association between the physical properties, chemical composition and the cooking quality of cowpea.

MATERIALS & METHODS

EIGHTEEN CULTIVARS of cowpeas (*V. unguiculata*) with 10–13% moisture were obtained from the National Cereals Research Institute, International Institute of Tropical Agriculture and a local market; all in Ibadan - Nigeria. The cultivars were grouped according to the coat texture, hilum color and coat color (Table 1).

Leached solids

One hundred seeds were cooked in about 250 mL boiling water for 50 min. The seeds were carefully removed and the cook-water was evaporated to dryness in a hot-air oven at 80°C overnight. The weight of the residue was taken and expressed in g% of the initial weight of the seeds before cooking.

The authors are with the Dept. of Human Nutrition, Univ. of Ibadan, Ibadan, Nigeria.

Table 1—External characteristics of 18 cowpea cultivars

Samples	Coat texture	Hilum color	Coat color
IT82D-716	Wrinkled	Brown	White-Brown
IT82D-889	Smooth	Black	Maroon
IT82E - 9	Smooth	Black	Brown
IT82E - 16	Smooth	Black	Maroon
IT82E-18	Smooth	Brown	Brown
IT82E-60	Wrinkled	Brown	Brown
K-17	Wrinkled	Brown	Brown
K-59	Wrinkled	Black	White
Nigeria B7	Wrinkled	Black	Brown
Nigeria A104	Wrinkled	Black	White
TVX 3236	Wrinkled	Brown	White-brown
Kano 1696	Wrinkled	Black	White
Farinjuda-C	Wrinkled	Black	White-grey
Ife-Brown	Wrinkled	Brown	Brown
Bauchi Crack	Cracked	Brown	Brown
Igbira	Smooth	Brown	White
Local Brown	Wrinkled	Brown	Brown
Local White	Wrinkled	Brown	White

Percent seed coat

Fifty seeds were weighed and soaked in 100 mL tap water for 3–12 min. The coats were carefully removed by hand, drained on filter papers (Whatman No. 1), weighed wet and re-weighed after drying to constant weight by the air-oven method (AOAC, 1980). The final weight was expressed as percent of total seed weight.

Seed density

One hundred seeds were weighed and then transferred into a 100 mL measuring cylinder containing 50 mL tap water. The seeds were allowed to soak for 10 min for equilibration and the volume of water displaced was recorded. The mass and the volume were then used in computing the density.

Swelling capacity

Fifty seeds of each variety were weighed followed by cooking in boiling water for 50 min. The seeds were drained and re-weighed. The final weights plus the leached solids were taken as the cooked weights. The swelling capacity was the difference between the raw weight and the cook weight per 100g seed.

Cooking time

The cooking time was estimated using a modified form of the bean cooker of Jackson and Varriano-Marston (1981). The plungers (38 ± 1.2g) were arranged with their needle-tips resting on the seeds previously soaked in 100ml water for 30 min. The cooking time was taken as the time the needles of 20 of the 40 plungers passed through the seeds. This was visible through the glass container.

Analytical Methods

The protein was estimated by the macro-Kjeldahl procedure (N × 6.25) (AOAC, 1980). The tannin was estimated by the vanillin-HCl method (Price et al., 1978) using catechin as standard. Soluble sugars were extracted with 85% warm ethanol (Southgate, 1969) and quantified by the phenolsulfuric acid procedure of Dubois et al. (1956). The amylose content was estimated using the rapid method of Juliano (1971).

Table 2—Physical properties of 18 cultivars of cowpeas

Cultivars	Cooking time (min)	Seed coat (%)	Leached solid (g%)	Seed density (g/cm³)	Swelling capacity (%)
IT82D-716	43	4.0	2.50	1.14	56.00
IT82D-889	36	14.0	1.20	1.18	89.50
IT82E-9	32	2.0	2.74	1.33	90.14
IT82E-16	51	15.0	0.64	1.22	91.14
IT82E-18	36	16.0	1.58	1.12	93.78
IT82E-60	45	3.5	0.29	1.31	85.69
K-17	75	6.6	3.23	1.10	108.63
K-59	53	4.7	0.47	1.20	100.47
Nigeria B7	106	4.6	4.03	1.17	108.23
Nigeria A104	87	6.0	1.30	1.16	94.40
TVX 3236	34	7.0	0.46	1.22	76.16
Kano 1696	50	5.4	2.62	1.09	99.22
Farinjuda-C	51	6.1	2.54	1.15	97.04
Ife Brown	55	1.5	1.41	1.24	107.71
Bauchi-Crack	32	6.8	5.73	1.10	122.23
Igbira	160	16.0	2.50	1.27	100.80
Local Brown	71	3.7	1.59	1.41	183.99
Local White	31	4.7	1.08	1.35	169.78
Mean ±	58.22	7.09	2.00	1.21	104.16
S.E.M.	±31.82	±4.60	±1.36	±0.09	±29.23

Statistical analysis

The mean and the standard error of mean were calculated for each physical property and the chemical composition for all the varieties and according to seed coat color, texture and hilum color (Tables 2, 3, 4). The correlation coefficients were computed for many pairs of the physicochemical parameters and the student t-test was used to determine the significance of the difference between the means at 95% confidence interval.

RESULTS

THE RESULTS (Tables 2 and 3) showed that the cooking time of cowpeas ranged from 31 min to 160 min, the seed coat was 1.5–16.0%, leached solids were 0.29–5.73%, and seed density 1.085–1.413 g/cm^3 (Table 2). The chemical compositions ranged from 0.24–0.40% for tannins, 6.30–13.9% for soluble sugars and 6.92–39.30% for amylose (Table 3). The mean values for the physicochemical characteristics are grouped according to the colors of the hilum and the seed coat and the seed coat texture (Table 4). Tvx 3216 and IT82D-716 (Table 1) were excluded because they had mottled grey-white and mottled brown-white seed coat, respectively. Bauchi crack was excluded because it had a peculiar cracked dark brown seed coat with "zebra" striation. The cooking times correlated positively to the crude protein, percent seed coat, swelling capacity, percent leached solids and the seed density (Table 5).

The correlation was negative for cooking time and amylose and for soluble sugars and tannins. Only the correlation between the soluble sugars and swelling capacity was statistically significant ($p < 0.05$). The results of the test of significance for the difference between the means among the groups showed that only the mean percent seed coat of the cultivars with smooth coats and those with wrinkled coats were significantly different ($p < 0.05$).

DISCUSSION

THE RESULTS of this study indicated that the degree of association between the physicochemical properties of cowpea and the cooking time varied with the parameters under comparison and the varieties studied. The thickness and the chemical composition of the seed coat, phytin, magnesium and calcium content have been associated with the cooking properties of seeds (Hambly, 1932; Muller, 1967; Longe, 1983). There was no significant difference in the cooking time between the smooth and wrinkled coat varieties in agreement with the report of Ojomo and Chheda (1972). However, while Ojomo and Chheda (1972) recorded a significant difference in the amount of water absorbed on soaking by the wrinkled (77.6g%) and the smooth (51g%) cultivars, no significant difference was observed in the swelling capacities of the two groups. This suggested that there was an initial barrier to water absorption which was eliminated on cooking. The poor linearity of the correlation between the cooking time, the swelling capacity and the leached solids suggested that some structural changes associated with cooking such as the denaturation of protein; gelatinization of starch and the disruption of the water transport barrier of the skin may play significant roles in the determination of the cooking time of cowpeas. A possible mechanism may involve initial lag, followed by a rapid relatively linear rate of change progressing to a saturation or equilibrium level. The decrease in the cooking time and the swelling capacity with increasing amylose content was contrary to the report of Tani (1958) that high amylose rice absorbed more water and swelled more during cooking than low amylose varieties. The high positive correlation between soluble sugar content and swelling capacity and the negative correlation between soluble sugar content and the cooking time suggested that the higher the sugar content the better the cooking quality. The lack of significant differences in the chemical and physical properties ($p > 0.05$) of cowpeas based on the seed coat (skin) colors showed that the color preference by Nigerian women did not have scientific basis. It could however, be explained on the basis of food habit and preference. However, the higher % seed coat recorded by the smooth coated varieties (11.19g%) than the wrinkled coated varieties (4.59g%) tended to justify

Table 3—Chemical composition of 18 cultivars of cowpeas

Cultivars	Crude protein [g% (N × 6.25)]	Soluble sugars (g%)	Amylose (g%)	Tannins (g%)
IT82D-716	22.85 ± 0.50	7.29 ± 0.33	12.25 ± 0.30	0.29 ± 0.02
IT82D-889	22.77 ± 0.86	7.40 ± 0.25	10.11 ± 0.53	0.30 ± 0.02
IT82E-9	21.90 ± 1.55	9.30 ± 1.71	31.43 ± 2.00	0.36 ± 0.03
IT82E-16	26.60 ± 0.77	9.32 ± 0.13	23.50 ± 0.64	0.27 ± 0.01
IT82E-18	25.72 ± 0.78	8.36 ± 0.45	8.46 ± 0.32	0.31 ± 0.04
IT82E-60	24.96 ± 6.50	8.00 ± 0.40	15.57 ± 0.20	0.31 ± 0.02
K-17	27.47 ± 0.60	10.20 ± 1.84	7.43 ± 0.80	0.40 ± 0.04
K-59	23.98 ± 1.39	11.27 ± 0.43	14.30 ± 1.10	0.33 ± 0.02
Nigeria B7	27.36 ± 0.31	7.83 ± 0.22	17.81 ± 0.21	0.38 ± 0.01
Nigeria A104	26.94 ± 0.64	8.25 ± 1.22	33.96 ± 1.06	0.34 ± 0.01
TVX 3236	24.30 ± 0.31	6.30 ± 0.12	39.30 ± 0.53	0.30 ± 0.01
Kano 1696	27.81 ± 1.24	12.16 ± 2.45	20.80 ± 0.40	0.33 ± 0.01
Farinjuda-C	27.47 ± 0.16	13.22 ± 1.22	21.70 ± 1.50	0.35 ± 0.07
Ife Brown	24.19 ± 1.60	12.50 ± 0.01	6.92 ± 0.42	0.33 ± 0.01
Bauchi-Crack	30.32 ± 0.47	13.58 ± 0.10	18.41 ± 1.20	0.35 ± 0.03
Igbira	27.37 ± 0.93	6.90 ± 0.02	9.34 ± 0.21	0.24 ± 0.02
Local Brown	26.38 ± 0.20	13.85 ± 1.00	12.35 ± 1.55	0.35 ± 0.02
Local White	24.56 ± 0.60	13.93 ± 1.22	15.45 ± 0.20	0.28 ± 0.04
	25.72 ± 2.12	9.98 ± 2.57	17.73 ± 9.11	0.32 ± 0.04

Table 4—Physicochemical properties of cowpeas (V. unguiculata) according to seed coat and hilium colors and the coat textures[a]

	Seed coat colors		Hilum colors		Coat textures	
	Brown (8)[b]	White (5)[b]	Black (8)[b]	Brown (10)[b]	Wrinkled (12)[b]	Smooth (5)[b]
Cooking time (min)	56.50 ± 24.39	76.20 ± 45.62	58.25 ± 23.70	58.20 ± 37.05	58.42 ± 21.31	63.00 ± 48.93
Leached solid (%)	2.58 ± 1.62	1.51 ± 0.84	1.94 ± 1.15	2.04 ± 1.51	1.79 ± 1.14	1.73 ± 0.04
Swelling capacity (%)	112.55 ± 29.22	112.93 ± 28.52	96.27 ± 5.35	110.48 ± 38.03	107.19 ± 24.57	93.07 ± 4.13
Skin (Coat) (%)	5.59 ± 4.32	6.96 ± 4.05	6.74 ± 4.08	6.52 ± 4.50	4.59 ± 1.40	11.19 ± 4.75
Density (g/cm^3)	1.22 ± 0.11	1.21 ± 0.09	1.19 ± 0.06	1.23 ± 0.11	1.21 ± 0.10	1.21 ± 0.08
Tannins (g%)	0.35 ± 0.03	0.30 ± 0.04	0.33 ± 0.03	0.32 ± 0.04	0.33 ± 0.03	0.30 ± 0.04
Soluble sugars (h g%)	10.45 ± 2.35	10.50 ± 2.57	9.84 ± 2.00	10.06 ± 2.91	10.38 ± 2.62	8.26 ± 0.98
Crude protein (g%)	26.04 ± 2.35	26.13 ± 1.56	25.60 ± 2.20	25.81 ± 2.05	25.69 ± 1.65	24.87 ± 2.15
Amylose (g%)	14.80 ± 7.60	18.85 ± 8.39	21.70 ± 7.53	14.52 ± 9.07	18.13 ± 9.44	16.57 ± 9.25

[a] Mean ± Standard deviation of mean.
[b] Number of the varieties that fall into each group.

Table 5—Correlation coefficients and the regression equations for the association between the physicochemical and cooking qualities of cowpeas

Y	X	Regression equations	Correlation coefficients
Cooking time	Crude protein	Y = 5.664X − 87.318	0.383
Cooking time	Amylose	Y = −0.679X + 70.245	−0.195
Cooking time	Soluble sugars	Y = −1.077X + 68.491	−0.114
Cooking time	Tannins	Y = −0.708X + 63.159	−0.277
Cooking time	Seed density	Y = 20.602X + 33.341	0.059
Cooking time	% Seed coat	Y = 1.698X + 46.185	0.246
Swelling Capacity	Cooking time	Y = 0.065X + 100.391	0.071
Swelling capacity	Soluble sugars	Y = 8.120X + 23.113	0.741*
Swelling capacity	Seed density	Y = 164.731X + 94.788	0.513*
Swelling capacity	Amylose	Y = −0.788X + 188.070	−0.244
Swelling capacity	% Seed coat	Y = −1.181X + 107.626	−0.137
Seed density	Crude protein	Y = −0.017X + 1.654	−0.404*
Swelling capacity	Crude protein	Y = 3.875X + 4.510	0.281
Leached solids	% Seed coat	Y = −0.045X + 2.299	−0.160
Leached solids	Swelling capacity	Y = 0.004X + 1.562	0.089
Leached solids	Cooking time	Y = 0.008X + 1.544	0.181

* Significant ($p < 0.05$).

their preference for the latter although their cooking times were not correspondingly different.

Of nutritional interest was the association between protein content and the cooking time. The cooking time increased with protein content. Although cooking increases the protein digestibility, prolonged cooking will increase the % leached solid and destroy the heat labile vitamins. Thus fast cooking does not only improve the acceptability of cowpeas but could also give the grains a higher nutrient retention during cooking by reducing the amount of leached solids.

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Kinetic Studies on the Alkaline Treatment of Corn (*Zea mays*) for Tortilla Preparation

MARINA HERRERA, JAYA ZYMAN, JORGE GARCÍA PEÑA, JOSÉ SIMÓN SEGURAJAÚREGUI, and JAIME VERNON

ABSTRACT

Water diffusion and starch gelatinization kinetics during corn nixtamalization were analyzed using the shell and core model. Good correlations were found between grain equivalent spherical radius and the phenomena involved: mass transfer ($r=0.97$, $P=0.001$) and reaction rate ($r=0.97$, $P=0.001$). A variable surface concentration model was used to explain calcium diffusion during processing. The parameters estimated allowed the simulation of the process at different cooking media temperatures and calcium hydroxide concentrations. The kinetic models developed in this work could be used to design, optimize and control the alkaline treatment of corn in preparing tortilla dough.

INTRODUCTION

NIXTAMALIZATION, an Aztec word meaning lime-cooked corn, is an ancient alkaline treatment used to soften the grain before grinding it into a dough which is then used in a variety of foods. In the traditional process, corn is slowly cooked in a hot lime solution and left overnight in the cooking solution until the pericarp is partially dissolved and loosened from the grain and the endosperm is swollen and softened. Both physical and chemical changes occur during cooking and soaking. Physical changes make grinding easier since swollen grains consume less energy during attrition milling and result in a smoother dough. Chemical changes, such as partial gelatinization of endosperm starch and denaturation of endosperm and germ proteins, give piable doughs.

Industrially, the traditional process has been empirically modified to minimize costs by reducing cooking and soaking times. For this purpose, grain:water and lime:grain ratios have been increased. The effects of lime:grain and water:grain ratios, cooking and soaking times, and grain variety on endosperm carbohydrates (Trejo-Gonzalez et al., 1982), dough quality (Bazúa et al., 1976) and finished product characteristics (Cravioto et al., 1945; Martínez-Herrera and Lachance, 1979) have been studied. Alternative cooking methods which have been suggested include corn flour extrusion (Bazúa et al., 1979), flake production by drum dying (Molina et al., 1977) and microwave grain drying (Smith et al., 1979).

Kinetic studies on simultaneous water diffusion and starch gelatinization have been developed for cooking rice prior to dehulling (Bakshi and Singh, 1980; Suzuki et al., 1976, 1977) and potato blanching (Kubota et al., 1979). Two kinetic models have been proposed for analyzing these phenomena: (1) Danckwerts' (1951) solution to simultaneous diffusion and reaction rates in a spherical particle; and (2) the shell and core model (Satterfield, 1970), which assumes symetric development of a gelatinized layer from the surface to the geometric center.

Author Segurajaúregui is with Depto. de Alimentos, División de estudios de Posgrado, Facultad de Química, Universidad Nacional Autónoma de México, 04510 México D.F. Authors Herrera, Zyman, Peña, and Vernon are with Area de Ingeniería Química, Departamento de Ingeniería de Procesos e Hidráulica, Universidad Autónoma Metropolitana, Unidad Iztapalapa, 03340 México D.F.

The lack of a rigorous analysis of the phenomena involved during corn nixtamalization justifies a study of the mass transfer and reaction rate kinetics. The objective of this work was to develop kinetic models for the rates of water and calcium diffusion and of gelatinization as a function of the cooking media temperature and calcium hydroxide concentration.

MATERIALS & METHODS

Raw material

An American yellow variety of commercial corn grain (*Zea mays L.*) was supplied by Compañía Nacional de Subsistencias Populares (CONASUPO).

Experimental procedure

To study water and calcium diffusion and gelatinization rates, experimental nixtamalization runs were conducted in duplicate at four different temperatures: 70, 80, 85, and 90°C, and two different calcium hydroxide concentrations: 1 and 2%. Twelve samples were prepared for each run in 250 mL Erlenmeyer flasks. Fifty grams of corn, 100 mL distilled water and either 1 or 2g of $Ca(OH)_2$ were placed in each flask. Flasks were covered with cotton and aluminium foil to avoid excessive evaporation of cooking water and immersed in a brine bath previously heated to the working temperature.

Analytical determinations

Flasks were withdrawn from the brine bath and the cooking water was drained. Corn grains were cooled and surface water absorbed with filter paper. Analytical determinations were performed in triplicate and the arithmetic means reported.

Corn grain radius. The grain apparent volume was measured by petroleum displacement in a 100 mL tube. From this value, the corn grain equivalent spherical radius was estimated.

Moisture was determined by drying the samples at 70°C and 48 mm Hg for 24 hr.

Calcium concentration was measured with a calcium electrode connected to a digital potentiometer. A standard curve was derived from control calcium carbonate solutions. Samples were prepared according to AOAC (1980) method 30.009.

RESULTS & DISCUSSION

Water diffusion and gelatinization rate

The shell and core model proposed by Suzuki et al. (1977) for rice cooking kinetics was used to analyze the kinetics of water diffusion and starch gelatinization in the corn grain. Figure 1 illustrates the model employed. This model assumes that the corn grain possesses a spherical geometry and that during nixtamalization the grain geometry increases due to internal water diffusion. As time proceeds, the nongelatinized fraction of the grain recedes internally until equilibrium is reached and all endosperm starch has been gelatinized. Therefore, at any time during the process, two regions can be identified: (1) a nongelatinized core, with radius r_c, and a moisture concentration equal to the initial grain moisture concentration, C_o, and (2) a gelatinized shell with a moisture concentration equal to the equilibrium moisture concentration, C_e. The rate of nixtamalization can be controlled by either the chemical reaction rate, the mass transport rate, or both phenomena as described below.

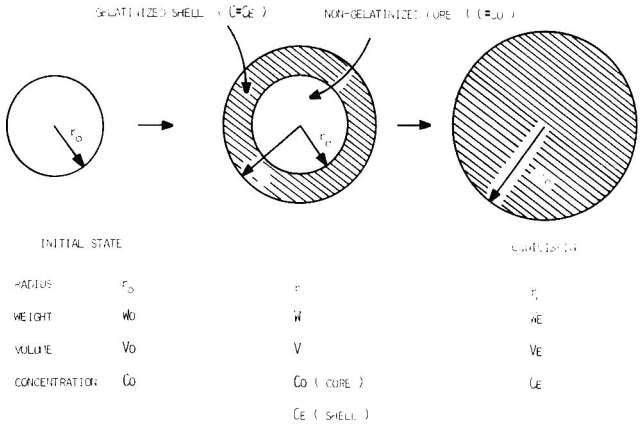

Fig. 1—Scheme of the shell and core model.

Reaction rate control

For first order kinetics,

$$-\left[\frac{dr_c}{d\theta}\right] = \frac{(r^2/r_c^2)\,\rho^{-1}\,(C_e - C_o)}{1/k_r\,(r_c^2/r^2)} \qquad (1)$$

where, $(dr_c/d\theta)$ is the rate of decrease of the nongelatinized grain fraction defined as the rate of decrease of the core radius, $(C_e - C_o)$ the moisture concentration difference in the core-shell interface, k_r the reaction rate constant, and ρ the initial grain density.

Mass transport control

$$-\left[\frac{dr_c}{d\theta}\right] = \frac{(r^2/r_c^2)\,\rho^{-1}\,(C_e - C_o)}{(r - r_c)/k_m(r_c/r)} \qquad (2)$$

where, k_m is the molecular diffusion coefficient.

Reaction rate and mass transport control

$$-\left[\frac{dr_c}{d\theta}\right] = \frac{(r^2/r_c^2)\,\rho^{-1}\,(C_e - C_o)}{\dfrac{1}{k_r\,(r_c^2/r^2)} + \dfrac{(r-r_c)}{k_m(r_c/r)}} \qquad (3)$$

As shown, the equivalent spherical radius change with time is directly proportional to the moisture concentration gradient at the core-shell interface and inversely proportional to the reaction rate and mass transport resistances: $1/k_r\,(r_c^2/r^2)$ and $(r-r_c)/k_m(r_c/r)$, respectively. The nongelatinized core radius, r_c, can be estimated using the following equation:

$$r_c = \left[\frac{r_e^3 - r^3}{(r_e^3/r_o^3) - 1}\right]^{1/3} \qquad (4)$$

where r_o is the initial grain radius, r the grain radius at any time, and r_e the grain radius in equilibrium with the surrounding calcium hydroxyde concentration and process temperature. To apply the shell and core model adequately, the magnitude of the equivalent spherical radius in equilibrium with the medium temperature and calcium hydroxide concentration must be determined for all experimental runs. For this purpose, the Langmuir model (Smith, 1970) was fitted to the experimental data:

$$r = r_o + \frac{k_l\,\theta}{1 + \dfrac{k_l\,\theta}{(r_e - r_o)}} \qquad (5)$$

where k_l is a model constant. Corn grain equilibrium radius

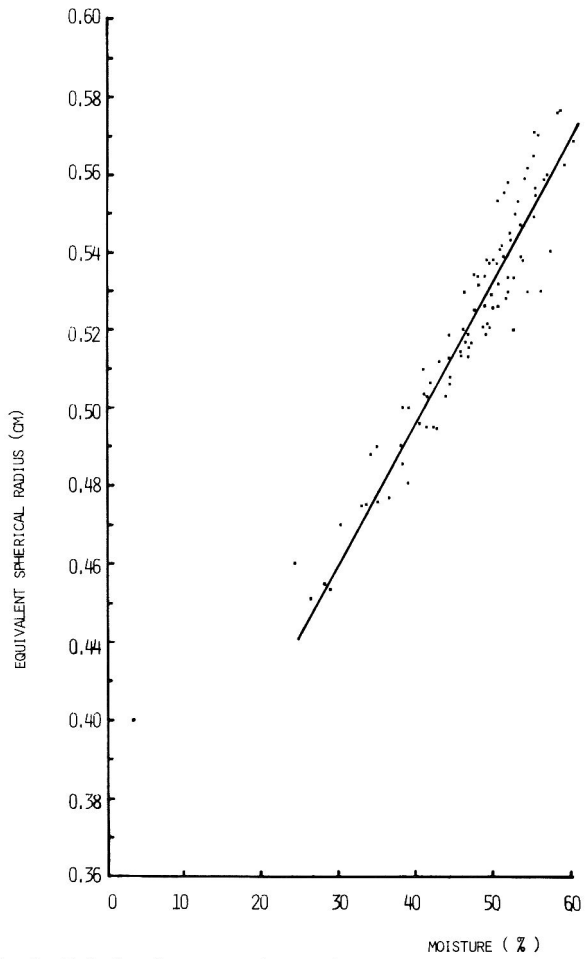

Fig. 2—Relation between the equivalent spherical radius and percent moisture. Regression equation: r= 0.34 + 4.0 × 10⁻³ (% H).

and its corresponding moisture content did not show a significant dependence on the process variables analyzed. Therefore, a mean equilibrium radius was employed for estimation of process parameters: r_e = 0.57 cm (%H = 57.03).

Analysis of water diffusion and gelatinization rate phenomena was based on the equivalent spherical radius change with process time. This interpretation can be justified by understanding the relation between equivalent spherical radius and the two physicochemical changes that take place during nixtamalization: an increase in grain moisture and starch gelatinization.

Figure 2 reports experimental data for equivalent spherical radius and their corresponding moistures for all the experimental runs. These two variables showed a linear dependence in the moisture range 25 to 60% (r = 0.97, P = 0.001). Results suggested that at the beginning of the operation water diffusion took place without a significant grain volume change and when the grain attained 25% moisture, radius changed linearly with moisture. The following linear correlation was obtained by the least squares method: $r = 0.34 + 4.0 \times 10^{-3}$ (%H), where r is the equivalent spherical radius (cm), and % H is the grain percent moisture, wet basis.

The degree of corn gelatinization during nixtamalization has been measured by several techniques: grain hardness (Martínez-Herrera and Lachance, 1979), viscoelasticity of the resulting dough (Trejo-Gonzalez, 1984) and differential alkaline solubility (Cabrera et al., 1984). These techniques require complex sample handling. A simple measurable variable which correlated with the phenomena involved during nixtamalization, corn grain radius change with time, could be used instead. Based on the results obtained by Cabrera et al. (1984), the

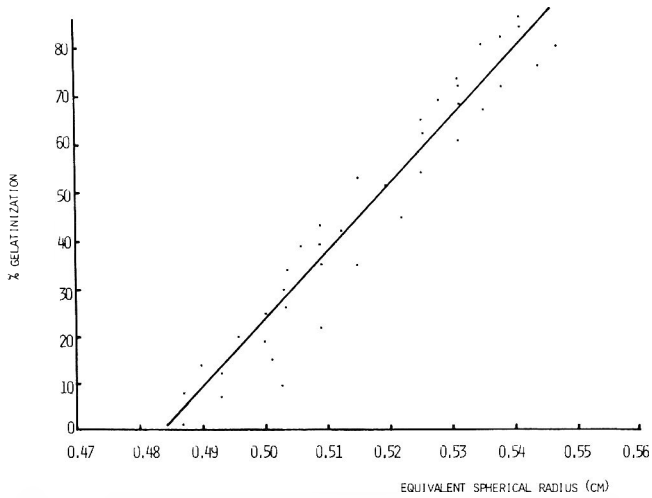

Fig. 3—Relation between the equivalent spherical radius and the percent gelatinization. Regression equation: % gel = −681.87 + 1410 r.

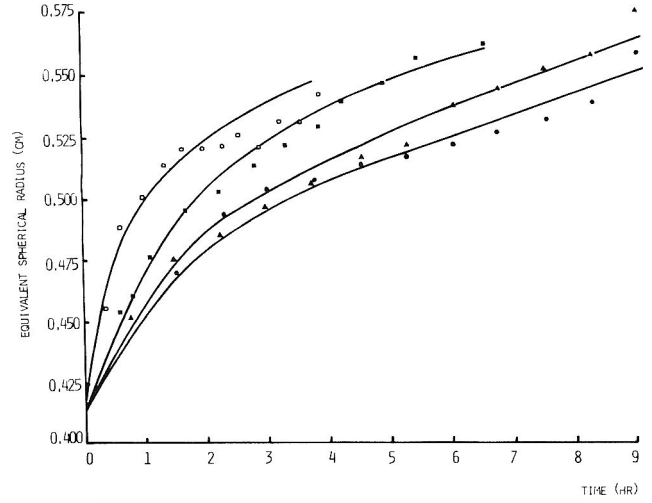

Fig. 4—Equivalent spherical radius change with time for a 1% $Ca(OH)_2$ concentration in the nixtamalization media. Working temperatures: 70°C (●), 80°C (▲), 85°C (■) and 90°C (□). Solid lines represent predicted values based on the shell and core model.

Table 1—Values obtained for the water diffusion coefficient, k_m, and the reaction rate constant, k_r, based on the shell and core model

	[Ca(OH)$_2$]			
	1%		2%	
Temp (°C)	k_m (cm^2/min)	k_r (cm/min)	k_m (cm^2/min)	k_r (cm/min)
70	5.0×10^{-4}	8.9×10^{-4}	8.3×10^{-4}	13.3×10^{-4}
80	6.7×10^{-4}	14.8×10^{-4}	10.0×10^{-4}	19.4×10^{-4}
85	10.0×10^{-4}	17.4×10^{-4}	13.3×10^{-4}	21.7×10^{-4}
90	15.0×10^{-4}	20.9×10^{-4}	16.7×10^{-4}	24.8×10^{-4}

relationship between degree of gelatinization and equivalent spherical radius during the alkaline treatment of a commercial Zoapila corn variety was analyzed. The results are shown in Fig. 3. Noticeably, a fair correlation ($r = 0.97$, $P = 0.001$) was found between these two variables.

Once the equilibrium equivalent spherical radius values were determined, the reaction rate and mass transport control equation (eq. 3) was solved numerically. Table 1 reports the results obtained for the reaction rate and mass transport parameters, k_r and k_m, respectively, and Fig. 4 and 5 show the variation of the average experimental radius with process time for 1 and

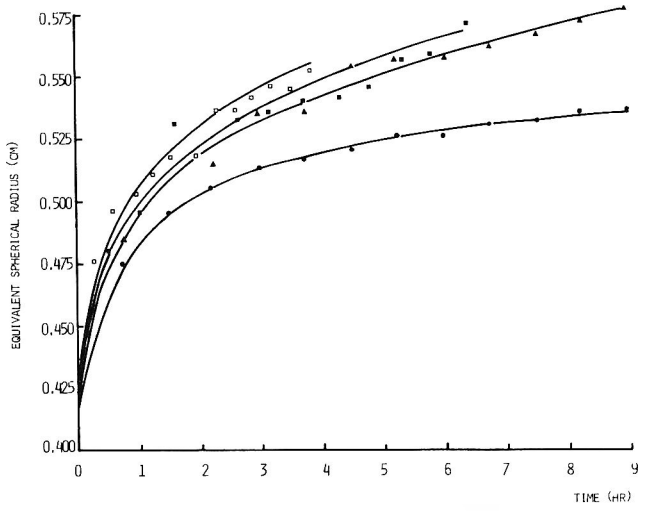

Fig. 5—Equivalent spherical radius change with time for a 2% $Ca(OH)_2$ concentration in the nixtamalization media. Working temperatures: 70°C (●), 80°C (▲), 85°C (■) and 90°C (□). Solid lines represent predicted values based on the shell and core model.

Table 2—Relative significance of the reaction rate and water diffusion resistances for T = 80°C and [Ca(OH)$_2$] = 1%

Time (min)	Chemical reaction resistance $R_1 = \frac{1}{k_r \cdot (r_c^2/r^2)}$ (cm/min)$^{-1}$	Internal mass transport resistance $R_2 = \frac{(r\text{-}rc)}{k_m \cdot (r_c/r)}$ (cm/min)$^{-1}$	R_2/R_1
40	0.284	0.043	0.15
90	0.355	0.074	0.21
135	0.406	0.095	0.23
180	0.448	0.112	0.25
225	0.517	0.139	0.27
270	0.604	0.170	0.28
315	0.658	0.189	0.29
360	0.994	0.292	0.29
405	1.377	0.398	0.28

2% calcium hydroxide concentrations in the cooking water at four different temperatures. The solid lines represent the radius-time profiles predicted by the proposed model. As can be seen, the shell and core model gives an accurate description of the geometrical changes exhibited by the corn grain during nixtamalization. Based on the correlations obtained between equivalent spherical radius and the physicochemical changes taking place in the grain during its processing, it can be concluded that the shell and core model explains adequately water diffusion and gelatinization phenomena.

Based on the magnitude of gelatinization rate and diffusion parameters, reaction and mass transport resistances, $(1/k_r(r_c^2/r^2)$ and $(r - r_c)/k_m$ (r_c/r), respectively, were evaluated. From these results, it was observed that gelatinization rate resistance controlled the rate of nixtamalization at all temperatures and calcium hydroxide concentration ranges studied. Nevertheless, the influence of diffusion resistance increased with the growth of the gelatinized layer. Table 2 shows the relative significance for both resistances at T = 80°C and [Ca(OH)$_2$] = 1%. These observations are in agreement with those of Suzuki et al. (1976, 1977) on rice cooking kinetics. On the other hand, as indicated by the magnitude of the transport and reaction rate parameters (Table 1), the water diffusion coefficient, k_m, is more sensitive to temperature than is the reaction rate constant, k_r. Therefore, an increase in media temperature resulted in a relative increase in the significance of diffusion resistance although cooking rate was still limited by the rate of gelatinization.

To develop a general model in terms of the operation variables, the dependence of the rate parameters upon cooking temperature and calcium hydroxide concentration was ana-

lyzed by multiple linear regression. The following equations were obtained:

$$k_m(cm^2/min) = 1.46 \times 10^{-2} - 4.01 \times 10^{-4}T + 2.9 \times 10^{-4}[Ca(OH)_2] + 2.8 \times 10^{-6}T^2 \quad (6)$$

$$k_r(cm/min) = -3.34 \times 10^{-3} + 5.15 \times 10^{-5}T + 4.3 \times 10^{-4}[Ca(OH)_2] + 4.09 \times 10^{-8}T^2 \quad (7)$$

Correlation coefficients exceeded 0.99 ($P = 0.001$) for mass transfer and reaction rate parameters equations.

Calcium diffusion

To explain internal calcium diffusion during the nixtamalization process, the variable surface concentration model was used (Crank, 1975). This model suggests that if the calcium concentration on the surface of a spherical geometry is a time function, $C'(\theta)$, the total amount of calcium entering the grain is given by the following expression:

$$\frac{C' - C_o'}{C_e' - C_o'} = 1 - \frac{3D}{\beta r^2}\exp(-\beta\theta)\left[1 - \left[\frac{\beta r^2}{D}\right]^{1/2} \cdot \cot\left[\frac{\beta r^2}{D}\right]^{1/2}\right] + \frac{6\beta r^2}{\P^2 D}\sum_{n=1}^{\infty}\frac{\exp(-Dn^2\P^2\theta/r^2)}{n^2(n^2\P^2 - \beta r^2/D)} \quad (8)$$

where C' is the grain calcium concentration at any time; C_o' the initial grain calcium concentration; C_e' the grain calcium concentration in equilibrium with the cooking medium; D is the calcium diffusion coefficient, a transport parameter which defines the surface calcium concentration variation and is given by

$$\frac{C_s' - C_o'}{C_e' - C_o'} = 1 - \exp(\beta \cdot \theta) \quad (9)$$

where C_s' is the surface calcium concentration. The value of the grain calcium concentration in equilibrium with the cooking medium was calculated from the Langmuir isotherm model (Smith, 1970):

$$C' = C_o' + \frac{k_l'\theta}{1 + \frac{k_l'\theta}{C_e' - C_o'}} \quad (10)$$

where k_l' is a model constant. Grain equilibrium calcium concentration dependence on process variables can be explained by the following equation:

$$C_e' \text{ (mg Ca/kg corn d.b)} = 3747.03 + 145.17[Ca(OH)_2] - 113.49T + 9.95T^2 \quad (11)$$

The variable surface concentration model is a nonlinear equation with two unknown parameters: β and D. This equation was solved by a nonlinear regression technique based on the Rosenbrock algorithm (Kuester and Mize, 1973). Nonlinear regression techniques are very sensitive to initial values for proposed parameters. For this reason, the initial value of β was estimated from the change in cooking medium calcium concentration with time, assuming that surface calcium concentration would vary in a corresponding way.

Values for the transport parameters β and D are shown in Table 3. To obtain a general equation for calcium diffusion the dependence of these parameters upon media temperature and calcium hydroxide concentration was determined by multiple linear regression analysis. The final equations are given below:

$$D\ (cm^2/min) = 0.80 + 3.02 \times 10^{-2}[Ca(OH)_2] - 2.22 \times 10^{-2}T + 1.53 \times 10^{-4}T^2 \quad (12)$$

$$\beta\ (min^{-1}) = 6.21 \times 10^{-2} + 2.16 \times 10^{-3}[Ca(OH)_2] - 1.71 \times 10^{-3}T + 1.17 \times 10^{-5}T^2 \quad (13)$$

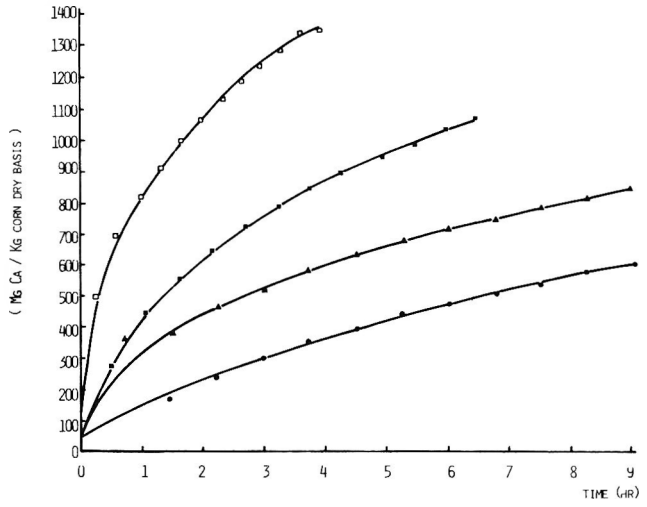

Fig. 6—Grain calcium concentration change with time for a 1% $Ca(OH)_2$ concentration in the nixtamalization media. Working temperatures: 70°C (●), 80°C (▲), 85°C (■) and 90°C (□). Solid lines represent predicted values based on the variable surface concentration model.

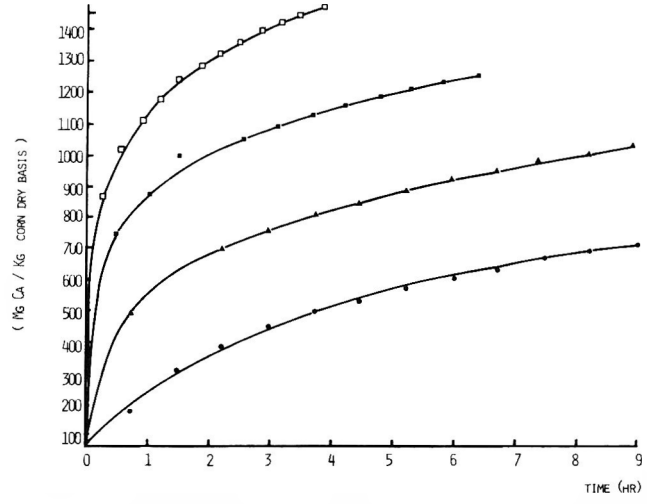

Fig. 7—Grain calcium concentration change with time for a 2% $Ca(OH)_2$ concentration in the nixtamalization media. Working temperatures: 70°C (●), 80°C (▲), 85°C (■) and 90°C (□). Solid lines represent predicted values based on the variable surface concentration model.

Table 3—Values obtained for the calcium internal diffusion coefficient: D, and surface concentration parameter, β, based on the variable surface concentration model

	[Ca(OH)₂]			
	1%		2%	
Temp (°C)	D (cm^2/min)	β (min^{-1})	D (cm^2/min)	β (min^{-1})
70	2.62×10^{-2}	1.93×10^{-3}	5.57×10^{-2}	4.18×10^{-3}
80	3.77×10^{-2}	2.83×10^{-3}	5.83×10^{-2}	4.18×10^{-3}
85	4.78×10^{-2}	3.50×10^{-3}	7.83×10^{-2}	5.70×10^{-3}
90	6.55×10^{-2}	4.78×10^{-3}	10.58×10^{-2}	7.80×10^{-3}

Correlation coefficients exceeded 0.98 ($P = 0.001$) for calcium transport parameters equations.

Figures 6 and 7 illustrate changes in grain calcium concentration with time as influenced by temperature and initial medium calcium hydroxide concentration. From the results obtained, it can be seen that both temperature and medium composition have a significant influence upon calcium internal diffusion.

SUMMARY & CONCLUSIONS

ALKALINE COOKING of corn was investigated in terms of the rate equations proposed by the shell and core model to explain water diffusion and gelatinization rate. Cooking rates were analyzed through corn radius measurements of alkaline treated corn exposed to different temperatures (70, 80, 85, and 90°C) and medium calcium hydroxide concentrations (1 and 2%). From the results obtained, it was concluded that cooking rate was limited mainly by the reaction of gelatinization, although the relative significance of diffusion increased both with increasing temperature and as cooking proceeded. Calcium diffusion was investigated through the variable surface concentration model. Analyzed process variables had a significant influence on calcium internal diffusion.

The kinetic models developed in this work could be used to simulate the traditional corn cooking process by solving the reaction rate and mass transport equation in terms of the specific thermal histories currently employed in the corn mill industry. Process simulation would lead to proper design, optimization and control of corn alkaline treatment for preparing tortilla dough.

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Heat Inactivation of the Ethylene-Forming Enzyme System in Cucumbers

HARVEY T. CHAN, JR.

ABSTRACT

Heat inactivation of the ethylene-forming enzyme system (EFE) in cucumbers was biphasic and both phases followed first order kinetics. The activation energies for the thermal inactivation of the heat resistant (HR) and the heat susceptible (HS) EFE's were 77.67 and 68.92 kcal/mole, respectively. The thermodynamic constants for the heat inactivation of both HS and HR are as follows: enthalapy, 68.27 (HS) and 77.03 (HR) kcal/mole; free energy; 21.2 (HS) and 22.9 (HR) kcal/mole; entropy, 146.1 (HS) and 168.2 (HR) cal/deg-mole. The heat resistant EFE appears to comprise 25-35% of the total EFE activity.

INTRODUCTION

HEAT TREATMENTS are being investigated as possible quarantine treatments for the disinfestation of fruit flies in a variety of fruits. The heat treatment developed for the disinfestation of papayas (Couey and Hayes, 1986) was shown to have deleterious effects on the ethylene-forming enzyme system (EFE) in papayas (Chan, 1986). The EFE is responsible for the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene (Adams and Yang, 1979). Heat inactivation studies on papaya EFE also showed the existence of two forms of EFEs with different heat resistances both of which were easily heat inactivated. Papaya EFE was also shown to be an excellent biochemical indicator of heat damage.

Cucumber (*Cucumis sativus* L.), as a host for the melon fly (*Dacus cucurbitae Coquillet*), is a likely candidate for heat treatment studies. Hence, knowledge of the heat sensitivity of cucumber EFE would help in the development of heat treatments for this fruit.

Because the EFE system is labile, possibly membrane bound (Imasaki and Watanabe, 1978; Apelbaum et al., 1981; Yang, 1985), and heat-sensitive (Yu et al., 1980) it might serve as a simple but effective biochemical indicator of fruit injury due to the heat treatments. In addition, a basic study of the thermodynamics of the heat inactivation of EFE may reveal some characteristics of the EFE system in general. This study reports the kinetics and thermodynamics for the inactivation of the EFE system in cucumbers.

MATERIALS & METHODS

Heat inactivation of fruit discs

Cucumber exocarp discs (10 mm diameter × 2.5 mm thick) were removed with a cork borer from cucumbers. The excised discs were placed into perforated plastic test tubes (30 mm diameter × 100 mm length), heated in a water bath for prescribed times and temperatures, and then cooled under running tap water (18–20°C).

Assay for EFE activity

EFE activity of excised disks was determined by following the conversion of administered ACC (Sigma Chemical Co.) to ethylene (Wang and Adams, 1982). Ten discs were incubated for 3 hr at 23°C with constant shaking in a sealed 25 mL Erlenmeyer flask that contained 15 mL 1 mM aminooxyacetic acid, 2% KCl, and 0 or 1 mM ACC. Three mL headspace gas was removed with a syringe and analyzed for ethylene by use of a Hewlett-Packard (Model HP5830) gas chromatograph equipped with a flame ionization detector. The carrier gas (helium) flow rate was 31 mL/min. The 1.2m × 2.3-mm i.d. stainless steel column was packed with Porapak QS 100/120 mesh. The column was operated isothermally at 60°C with the detector temperature at 250°C and the injector at 150°C.

EFE activity was expressed as nL of ethylene produced/g fresh weight-hr.

Calculation of thermodynamic constants

The thermodynamic equations of Glasstone (1960) and Whitaker (1972) were used to calculate the first order rate constants, k, the energy of activation, E_a, enthalapy, H, free energy, F, and entropy, S. The rate constants were calculated by iteratively fitting the following equation to the observed data points:

$$\text{EFE activity} = p\, e^{-k't} + (100\text{-}p)e^{-k''t} \qquad (1)$$

where p is the percent of the postulated heat resistant enzyme, k′ and k″ are the rate constants of the heat resistant (HREFE) and heat sensitive (HSEFE) ethylene-forming enzyme, respectively, and t is the exposure time.

RESULTS & DISCUSSION

HEAT INACTIVATION studies of plant enzymes are normally performed in-vitro on partially or highly purified enzyme extracts in capillary tubes or thin-walled test tubes to minimize problems due to heat transfer. However, attempts to produce an active EFE extract in this laboratory have been unsuccessful; there also are no reports in the literature on the successful extraction and isolation of EFE. Intact membranes appear to be a requisite for EFE activity (Lurssen et al. 1979; Guy and Kende, 1984). Hence, discs of cucumber flesh were used in this study. A plot of log % EFE activity in cucumbers vs heating time resulted in curves rather than straight lines (Fig. 1). If the thermal inactivation of EFE followed first order kinetics, a straight line would have been obtained for each temperature. Thus, our initial conclusion was that the thermal inactivation of cucumber EFE did not follow first order kinetics. However, further inspection of the inactivation curves indicated the existence of a composite curve for each temperature which could be resolved graphically into two straight lines. A graphic resolution was performed for the inactivation curve at 46°C and for the inactivation curves at the other temperatures (Fig. 2). The existence of two straight lines for each inactivation curve indicated the presence of two types of EFE, of which one type is more heat resistant than the other. The existence of two types of EFE systems with different sensitivities to heat treatments has been previously reported for papayas (Chan, 1986).

The existence of biphasic first order curves in other enzyme systems have generally suggested either the presence of isozymes, as for papaya invertase (Chan and Kwok, 1976), papaya catalase (Chan et al., 1978), papaya acid phosphatase (Carreno and Chan, 1982), and corn peroxidase (Yamamoto et al., 1962), or the presence of multiple forms of enzymes, as in the case of papaya exo- and endo- polygalacturonases

Author Chan is with the USDA-ARS, Tropical Fruit & Vegetable Research Laboratory, P.O. Box 4459, Hilo, HI 96720.

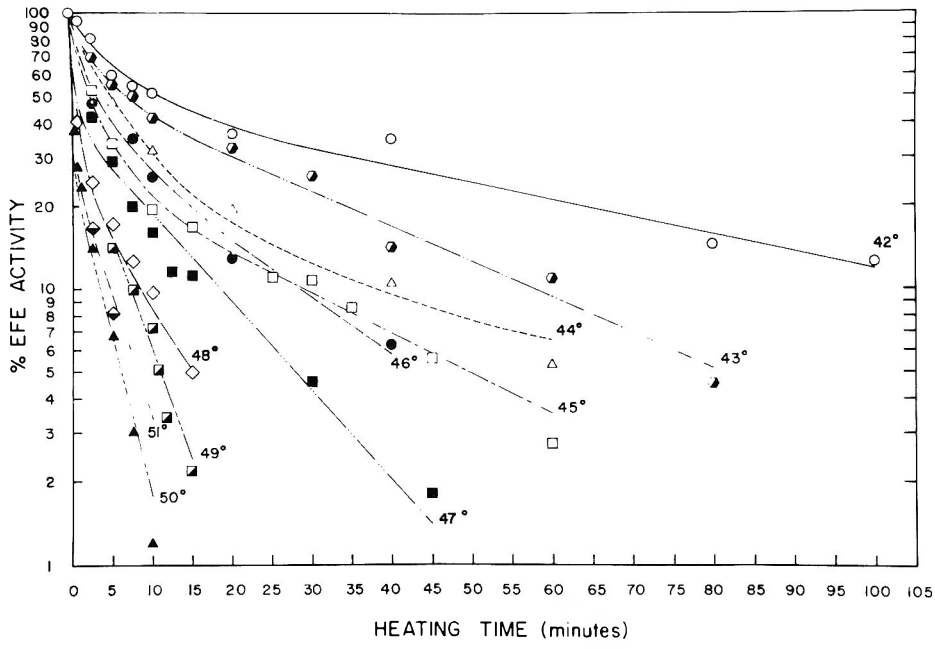

Fig. 1—Log EFE activity (%) as a function of temperature. The means of three replications are shown. Some of the data points for temperatures > 44°C and < 5 min were not be presented graphically due to limitation of space.

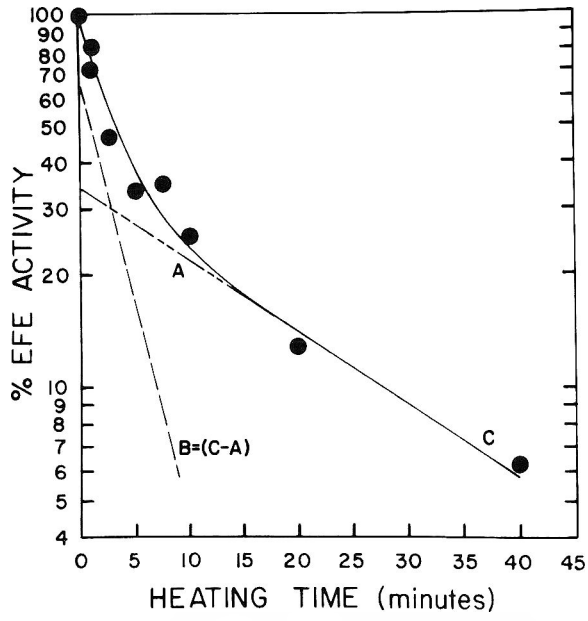

Fig. 2—Graphic resolution of the composite heat inactivation curve [log EFE activity (%) at 46°C] for cucumber (EFE) ethylene-forming enzyme. A = heat resistant ethylene-forming enzyme (HREFE) portion; B = heat sensitive ethylene forming enzyme (HSEFE) portion; C = remaining total EFE activity.

(Chan and Tam, 1982). In the case of the EFE system, the interpretation of the presence of biphasic thermal inactivation curves must be restricted at this time to only the existence of at least two forms of EFEs with different heat resistances. The existence of EFE isozymes or other EFE systems such as the nonstereospecific and stereospecific EFE (McKeon and Yang, 1984) can only be confirmed after the EFE system has been successfully isolated and characterized. The high coefficients of determination, R^2, indicate that the pseudo first order kinetics model (Eq. 1) effectively described the heat inactivation of the EFE system in cucumbers (Table 1). Attempts to fit zero- and second-order models were not successful. Further evidence for the validity of the pseudo first order kinetics model was provided when linear Arrhenius plots were obtained for both HSEFE and HREFE (Fig. 3). The rate constants (k) and the D values for the two forms of EFE are listed in Table 1.

The stability of the enzymes is presented as D values (Table 1), i.e. decimal reduction times, which are defined as the time required for 90% destruction of enzyme activity at constant temperature (Yamamoto et al., 1962). The D values for the HREFE were considerably larger (>12 X) than those for the HSEFE, which indicate that much longer heating times were required for the heat inactivation of HREFE.

An extrapolation of the thermal inactivation curve for the HREFE at 46°C to zero heating time indicated that the heat resistant portion comprises about 34.0% of the total EFE activity, with the heat susceptible portion accounting for the remaining 66.0% (Fig. 2). The activation energies (E_a) for thermal inactivation of the HREFE and the HSEFE were 77.67 and 68.92 kcal/mole, respectively. The higher E_a for the inactivation of the HREFE is significant, because, according to the Arrhenius equation, small changes in temperature will cause large changes in the reaction-rate constant, k, or in the rate of heat inactivation. Hence the Q_{10} for the HREFE was 1.54 times as large as the Q_{10} for the HSEFE. The free energies, ΔF, for both the HREFE and HSEFE of cucumbers were in the range (20–30 kcal/mole) considered by Eyring and Stearn (1939) to be characteristics of thermal denaturation of proteins (Table 1). The entropy, ΔS, for the HREFE and the HSEFE had a large and positive value. Such large positive values for ΔS suggest that the inactivation of both HREFE and HSEFE was accompanied by an unfolding of either the polypeptide chain of the enzyme or of its membrane matrix into a more random structure. The higher ΔS for the HREFE than for the HSEFE also implies that the HREFE undergoes a greater change in structure in its transition state than the HSEFE.

The number of noncovalent bonds broken on denaturation of a protein can be estimated if an average ΔH of 5 kcal/mole is used per bond (Whitaker, 1972). By dividing the enthalpy (ΔH) of each EFE by 5 kcal/mole the number of noncovalent bonds broken on the HSEFE and HREFE were 13.7 and 15.4 bonds, respectively. Hence the HREFE was most likely in a more ordered state than the HSEFE because it had more noncovalent bonds stabilizing its native structure. The high entropy change for HREFE was due to a greater loss of order commencing from a more ordered state than the HSEFE and resulting in a similar state of disorder. A comparison of the D values at 49°C for cucumber HSEFE (1.29 min) and papaya HSEFE (9.52 min) (Chan 1986) shows that the EFE system in cucumbers is much more sensitive to heat treatment than that in papayas. This greater sensitivity may explain the greater

Table 1—Kinetics data for heat inactivation of cucumber EFE

Temp °C	Rate constant, min^{-1}			D value[a], min	
	Heat susceptible	Heat resistant	R^{2b}	Heat susceptible	Heat resistant
42	0.164	0.009	0.974	14.01	234
43	0.232	0.014	0.993	9.89	160
44	0.328	0.020	0.983	7.01	110
45	0.463	0.030	0.999	4.97	75.5
46	0.651	0.044	0.918	3.53	52.1
47	0.914	0.064	0.974	2.52	36.0
48	1.28	0.092	0.956	1.80	24.9
49	1.79	0.133	0.988	1.29	17.3
50	2.49	0.191	0.974	0.922	12.0
51	3.47	0.275	0.969	0.663	8.37
E_a[d]	68.92 kcal/mole	77.67 cal/mole			
Q_{10}	30.38	46.87			
H	68.27 Kcal/mole	77.03 Kcal/mole			
F	21.21 Kcal/mole	22.88 Kcal/mole			
S	146.1 e.u.	168.2 e.u.			

[a] Decimal reduction times

[b] R^2 = coefficient of determination

[c] Calculated

[d] E_a is the energy of activation; Q_{10} is the temperature coefficient; ΔH is the enthalapy; ΔF is the free energy; ΔS is the entropy; e.u. is entropy units = cal/degree-mole.

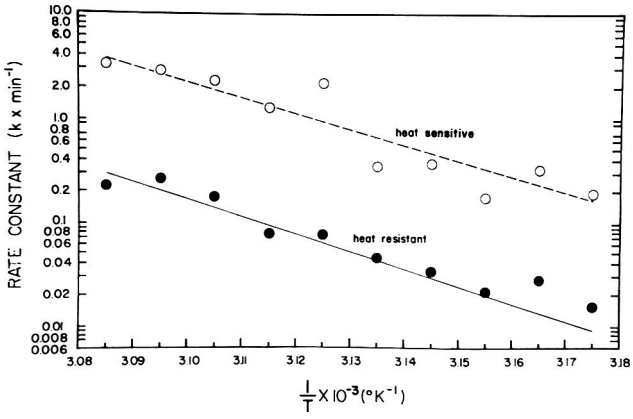

Fig. 3—Arrhenius plot of rate constant, k, as a function of temperature for the heat inactivation of the heat resistant and heat sensitive (EFE) ethylene-forming enzyme in cucumber.

difficulties in developing nonphytotoxic heat treatments for cucumbers.

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Concentration of Perilla Anthocyanins by Ultrafiltration

MEI YUH CHUNG, LUCY SUN HWANG, and BEEN HUANG CHIANG

ABSTRACT

Anthocyanins were extracted from perilla leaves using 10% citric acid solution. The pigment in the extract could be concentrated by ultrafiltration (UF) with a membrane of M. W. cutoff 6,000, possibly due to the formation of copigmented complexes. In UF operations, fouling was not severe. Increasing either transmembrane pressure or feed concentration resulted in increased solute retention. Recovery of anthocyanin was over 60% by UF at volume concentration ratio of 4.

INTRODUCTION

PERILLA (*Perilla ocymoides* Linn.) which originated in China also grows quite well in Taiwan and Japan. The leaves of perilla contain large quantities of anthocyanins, estimated to be up to 1.9g per 100g dried leaves (Huang and Hwang, 1980). The anthocyanins are cyanidin-3,5-diglucosides acylated with coumaric acid or caffeic acid (Jadot and Niebes, 1968; Wu and Hwang, 1980; Ishikura, 1981). Since the color of perilla anthocyanins is very similar to amaranth (FD&C Red No. 2), it has been used for coloring pickled plums in Japan. Currently, there is an increasing demand for perilla anthocyanins as food color in Japan (Ishikura, 1981).

Anthocyanin extracts are usually concentrated by vacuum evaporation (Baker et al., 1974; Nedelchev et al., 1975; Ferenczi and Kerenyi, 1979). However, natural anthocyanins are, in general, extracted more efficiently with stronger acidic solutions (Metivier et al., 1980; Chung, 1984). Thus, the concentration of the pigment would be limited by the rapid build up of total solids.

The object of this study was to investigate the concentration of perilla anthocyanins in citric acid solution by ultrafiltration. It was hoped that the membrane could retain the pigment while allowing citric acid to pass through, so that the solids content of the solution would not limit the concentration ratio of the pigment. The major factors considered in this investigation were the solute retentivities and the permeation flux during membrane processes.

MATERIALS & METHODS

Preparation of pigment extract

Perilla (*Perilla ocymoides* Linn. var. crispa Benth. form purpurea Makino) leaves were dried in an air blast drier at 50°C for approximately 7 hr until moisture was reduced to 10%. The dried leaves were ground by hand and the anthocyanins were extracted with aqueous 10% citric acid. Seven-hundred grams of dried leaves were soaked in 10L of solvent, and the mixture was left standing for 24 hr at 25 ± 2°C. The extract was drained and reused as solvent for three further extractions. The extracts produced by this method were blended in a large storage tank and stored at 4°C.

Equipment

The De Danske Sukkerfabrikker (DDS) Lab Module-20 (Copenhagen, Denmark) was used for ultrafiltration (UF) and reverse osmosis (RO) studies. The module was a plate-and-frame system which contained four membrane plates with effective area of 0.072 m^2. In addition to the above unit, the Romicon HFXS-5/10 hollow fiber unit (Woburn, MA) was also used for UF process. Each cartridge provided an effective membrane area of 0.1416 m^2. Specifications for the membranes used are given in Table 1.

Authors Hwang and Chiang are affiliated with the Graduate Institute of Food Science and Technology, National Taiwan Univ. Taipei, Taiwan, ROC. Author Chung, formerly with the National Taiwan Univ., is now affiliated with the Wei-Chuan Foods Co., 125, Sung Chiang Rd., Taipei, Taiwan, ROC. Address inquiries to Dr. Chiang.

Table 1—Specifications of membranes for ultrafiltration and reverse osmosis

Membrane	Permeability[c]	Flux (L/hr/m^2) (measured at 20°C with various pressures)	Max operation pressure, MPa
RO membrane[a]			
DDS HR 95	5	76 (4.2 MPa)	8.0
DDS HS 65PP	70	150 (3.0 MPa)	3.0
UF membrane[b]			
DDS GR 81PP	6000	100-250 (0.5 MPa)	1.5
Romicon HF 5-43 – PMI	1000	40 (0.2 MPa)	0.276

[a] RO membranes tested on 0.25% NaCl solution

[b] UF membranes tested on pure water

[c] For UF membrane the nominal MW cut-off values are given. The % permeabilities for 0.25% NaCl solution are given for RO membrane.

Membrane processes

In this study, all tests were conducted at 25°C. The feed flow rate was fixed at 7.3 L/min for the plate-and-frame UF and RO operations. For the fouling studies, both permeate and retentate were recycled to the feed tank to maintain constant feed concentration. At various transmembrane pressures (ΔP_T), $\Delta P_T = (P_{inlet} + P_{outlet})/2$, the changes of flux were recorded over a 3.5 hr operating period to determine the flux decay due to fouling. The process performance was studied after operation of the unit for approximately 2 hr, when the phenomenon of flux decline was eased. The performance was evaluated in terms of effect of feed concentrations and operating pressures on permeation rate.

Retention characteristics of the membrane

Samples of permeate and feed solution were collected at various operating conditions. Each sample was analyzed for total solids and anthocyanin concentration. The retention characteristics were evaluated by calculating the retentivity (R) and percent solute recovery according to the following formulas:

$$R = (1 - \frac{C_f}{C_b}) \times 100\%$$

where C_f = solute concentration in permeate, C_b = solute concentration in feed.

$$\% \text{ solute recovery} = \frac{C_t V_t}{C_i V_i} \times 100\%$$

where C_t = solute concentration at time t, C_i = initial solute concentration, V_t = volume of retentate at time t, V_i = initial volume of the feed.

Chemical analysis

Total solids were determined by drying 10 mL of sample in a 40°C vacuum oven to constant weight. Soluble solids were determined by measuring the refractive index (n_D) with a refractometer (AO ABBE Refractometer, Model 10450, Buffalo, NY) at 25°C after centrifuging the sample at 1000 rpm for 15 min. The soluble solids were then determined using a standard curve which was prepared by plotting n_D against soluble solids of samples with known concentrations. The Kjeldahl procedure was used to analyze for total nitrogen. A factor of 6.25 was applied to estimate the crude protein content (AOAC,

Table 2—Composition of the perilla leaves extract

Crude protein	0.246 ± 0.008 %
Pectin	0.093 ± 0.003 %
Ash	0.175 ± 0.011 %
Soluble solids	9.98 ± 0.36 %
Total solids	10.66 ± 0.36 %
Anthocyanin	0.016 ± 0.002 %

1980). Total ash was determined by dry ashing the sample at 550°C. Pectin was determined by precipitating the pectic substances from the sample solutions with ethanol followed by dissolving the precipitates in water and quantitated by chelatometric titration (Huang *et al.*, 1967).

A modified pH differential method was used to determine the anthocyanin content (Fuleki and Francis, 1968a, b). The acidic aqueous solution of perilla anthocyanins was found to have a λ_{max} at 520 nm and showed minimum absorbance at this wavelength when the pH was adjusted to 5.5. Therefore, the absorbance at 520 nm (A_{520}) of the sample was measured at pH 1.0 and 5.5 after proper dilution with deionized distilled water and adjustment of pH with conc HCl or NaOH solutions. A UV-VIS Spectrophotometer (Shimadzu Double Beam Spectrophotometer, Model UV-200S, Japan) was employed and the analysis was carried out in duplicate. The anthocyanin content was calculated by the following equation:

$$\text{Anthocyanin content (g/L)} = \frac{A \times F \times MW}{\epsilon}$$

where $A = A_{520}$(pH 1.0) − A_{520}(pH 5.5); F = dilution factor; MW = molecular weight of anthocyanin, 811, the MW of the major perilla anthocyanin, cyanidin-3-p-coumaryl-5-diglucoside; ϵ = molar absorptivity, 3.0175×10^4, the ϵ of cyanidin-3,5-diglucoside in aqueous solution was used in this study (Niketic-Aleksic and Hrazdina, 1972). Since % anthocyanin retention was calculated in most of the data presented in this study, the values of MW and ϵ were cancelled during calculation.

The visible λ_{max} of perilla extract was measured at various concentrations with 10% citric acid solution (the extracting solvent) to determine if the phenomenon of copigmentation occurred in the perilla extract. The visible λ_{max} of the undiluted extract was measured in a 1.0 mm path length glass cuvette. The 2,3.3 and 12.5-fold diluted extracts were measured in glass cuvettes with 2, 3 and 10 mm path length, respectively.

RESULTS & DISCUSSION

CHUNG (1984) has investigated the efficiency of various acidic ethanols and acidic aqueous solutions in extracting perilla anthocyanins from the dried leaves. Similar to the results reported by Metivier et al. (1980) for grape pomace, Chung (1984) found that 10% HCl in ethanol was the most efficient solvent. However, it was found that acidic ethanols also extracted the undesirable chlorophyll pigments from the perilla leaves. Acidic aqueous solutions could extract anthocyanins from dried perilla leaves faster than the acidic ethanols, and they also avoided the extraction of chlorophyll pigments. Aqueous solutions containing either 10% hydrochloric acid or citric acid showed similar effectiveness for the extraction. Since 10% hydrochloric acid may cause hydrolysis of anthocyanins during prolonged processing, 10% citric acid solution was suggested (Chung, 1984).

The proximate composition of the extract is given in Table 2. The high percent soluble solids limits concentration of the extract by evaporation to approximately six times achieving a final anthocyanin content of no more than 0.1%. This suggested that separation of the citric acid was essential for the production of the colorant concentrate. Membrane process is a technique which can fractionate and concentrate components in solutions based on molecular size differences. Although the molecular weight difference between anthocyanin (MW range around 800) and citric acid (MW = 250) is not great, UF and RO are possible methods for the separation and concentration. The feasibility of a number of membranes was investigated. The DDS HR-95 RO membrane was found to retain nearly 100% of the anthocyanin but resulted in low flux (Fig. 1). The

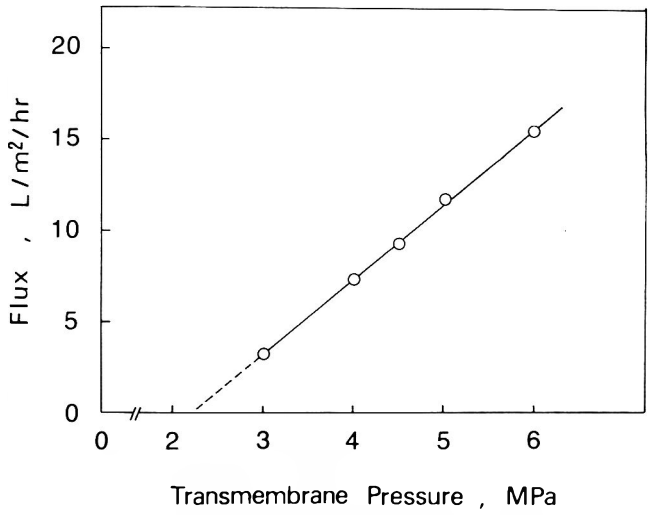

Fig. 1—Relationship between permeate flux and operating pressure of RO process using HR-95 membrane.

low permeation rate was believed to be due to the high osmotic pressure of the citric acid in the solution. Since the relationship between pressure and flux was linear, extrapolating of the line to zero resulted in an estimated osmotic pressure of approximately 22 bar for the feed. This high osmotic pressure made this membrane unsuitable for the intended purpose.

The DDS HS65PP membrane (M.W. cut-off = 500) should retain anthocyanin while allowing citric acid to pass through. This property would eliminate the osmotic pressure problem caused by the high concentration of the citric acid in the feed solution. The results showed that the flux of this membrane was very poor. The permeation rate at 2.8 MPa operating pressure was below 4.8 L/m^2 hr. A similar test was conducted using the Romicon PM-1 UF membrane (M.W. cut-off = 1000) on Romicon HFXS-5/10 hollow fiber unit, but the flux was practically zero at maximum allowable operating pressure.

Since membranes with low molecular weight cut-off did not provide enough flux for concentrating the extract, UF membrane with higher degree of porosity was tested. A high M.W. cut-off membrane might give satisfactory results because the permeation rate of UF operation often increases with increase in membrane porosity. Besides, anthocyanins also may exist as copigmented complexes rather than single molecules (Asen et al., 1972). The copigmented complexes may facilitate the retention of the colorant with high porosity UF membrane.

Retention characteristics of high porosity UF membrane

Figure 2 gives the retention characteristics of the DDS GR81PP UF membrane (M.W. cut-off = 6000) for anthocyanins and total solids at various concentrations. A significant difference in retentivities between anthocyanins and other solutes was observed. This finding indicated that the perilla anthocyanins might form copigmented complexes in citric acid solution, and a great portion of the pigment complexes were large enough to be retained by UF membrane of molecular weight cut-off of 6000. Ishikura (1981) found that perilla has two flavones and nine flavone glycosides which can act as copigments. In this study, the existence of copigment complexes in the extract was further demonstrated by a shift of the visible absorption maximum (λ_{max}) after the extract was diluted. The λ_{max} of the undiluted extract showed a 17 nm bathochromic shift from the 12.5-fold diluted extract which had a λ_{max} at 524 nm. In addition, as the concentration of anthocyanins increased, the magnitude of the bathochromic shift also increased (Table 3). This increase of bathochromic shift may also be due to the fact that the less diluted extract had a more favorable pH for copigmentation (Table 3). Williams and

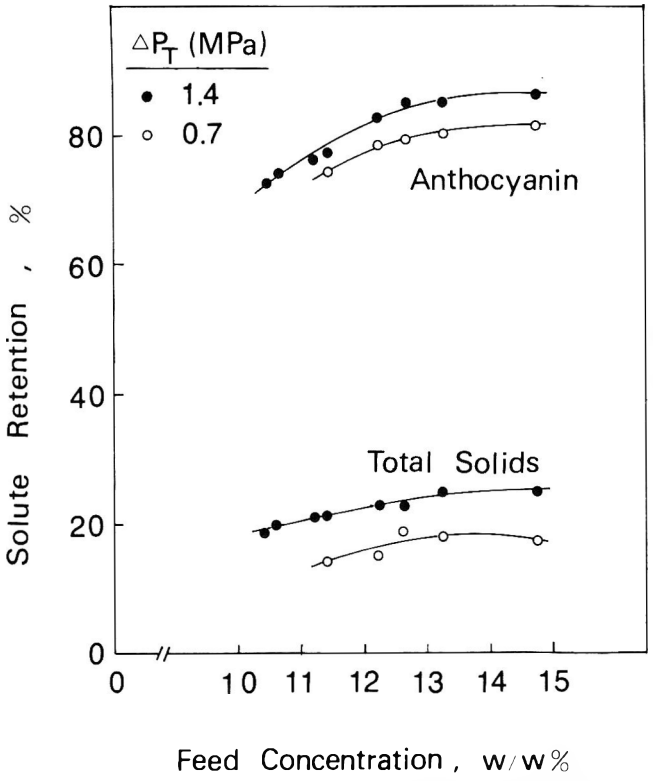

Fig. 2—Effect of feed concentration on solute retention at various transmembrane pressure, using GR81PP membrane.

Table 3—Visible λ_{max} of perilla anthocyanins in undiluted and diluted extracts[a]

	Undiluted	2-fold dilution	3.3-fold dilution	12.5-fold dilution
pH	2.86	2.46	2.3	1.8
λ_{max}(nm)	541	536	531	524

[a] The solvent was 10% citric acid solution.

Hrazdina (1979) reported that for the 3-acylglucoside-5-glucosides of anthocyanins the maximum copigmented complex formation was around pH 3.1. In the membrane process of this study, the feed solution (undiluted extract) had a pH value of 2.86 which remained unchanged throughout the UF process. Therefore, the existence of anthocyanin copigmented complexes under this favorable pH condition is believed to be one of the reasons why UF membrane of MW cut-off 6000 can retain perilla anthocyanins. Retention of anthocyanin was also found to increase as the concentration increased (Fig. 2). It has been reported that copigmented complex formation was affected by the concentration of anthocyanin in the solution (Asen et al., 1972; Scheffeldt and Hrazdina, 1978). Results of this study suggested that more copigmented complexes may be formed at high concentration.

Figure 3 illustrates the relationships between operating pressures and solute retentions. The pressure increase resulted in an increase in retentivity. Similar results with various other systems were reported by Fenton-May et al. (1971), Nguyen et al. (1980), and Lee (1981). The increased retentivity was attributed to the increased compaction of membrane and the gel layer on the surface of the membrane at high pressure.

Processing characteristics

The major concern of UF processing is the fouling phenomena after prolonged operation time. In this study, flux decay of processing perilla leaves extract by the plate-and-frame UF system was not very severe as shown in Fig. 4. The permeate flux dropped rapidly in the first 30 min, after which it became

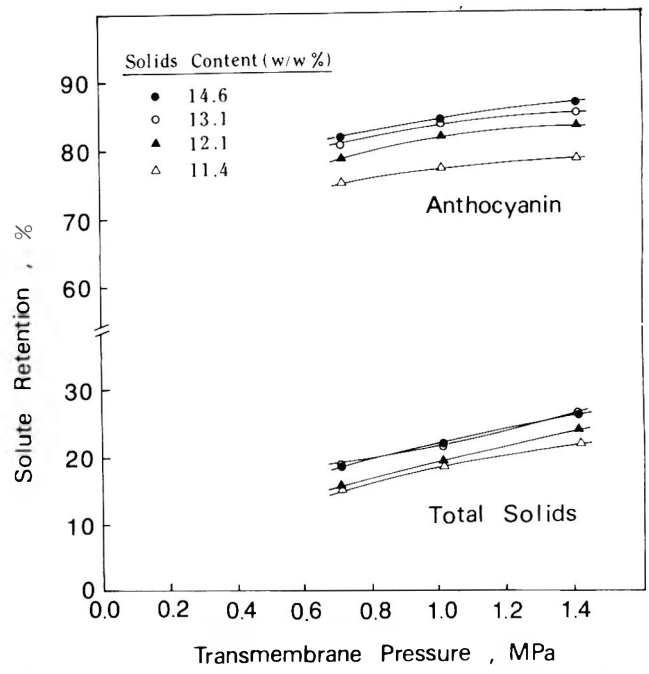

Fig. 3—Effect of transmembrane pressure on solute retention at various feed concentrations, using GR81PP membrane.

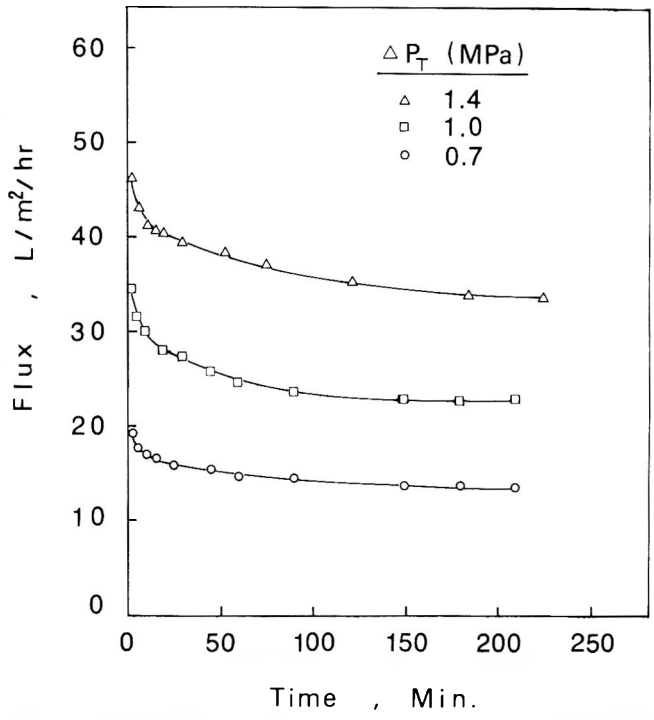

Fig. 4—Flux decay as a function of processing time for GR81PP membrane at various transmembrane pressures. Feed flow rate was at 7.3 L/min.

relatively steady. Kuo and Cheryan (1983) studied the fouling phenomena of cheese whey in spiral wound system using the mathematical model, $J_t = J_1 t^{-b}$, where J_t is the flux at time t, J_1 is the instantaneous flux at t = 1 min and b is the fouling index. It was reported that at low flow rate (3 L/min), pressure had little or no effect on b values. But at high flow rate (10-11 L/min), too high a pressure (485 KPa), the rate of fouling became very great. According to the fouling theory the authors proposed, high flow rates bring more solids to the membrane surface where high pressure compresses the rejected solutes into a thicker and denser hydraulic barrier. In this study, the

Table 4—Effect of transmembrane pressure on b values of equation $J_t = J_1 t^{-b}$. (J_t is the flux at time t, J_1 is the flux at t = 1 min)

Transmembrane pressure (MPa)	b values	r[a]
1.4	0.066	0.990
1.0	0.086	0.986
0.5	0.053	0.966
0.4	0.067	0.991

[a] Correlation coefficient

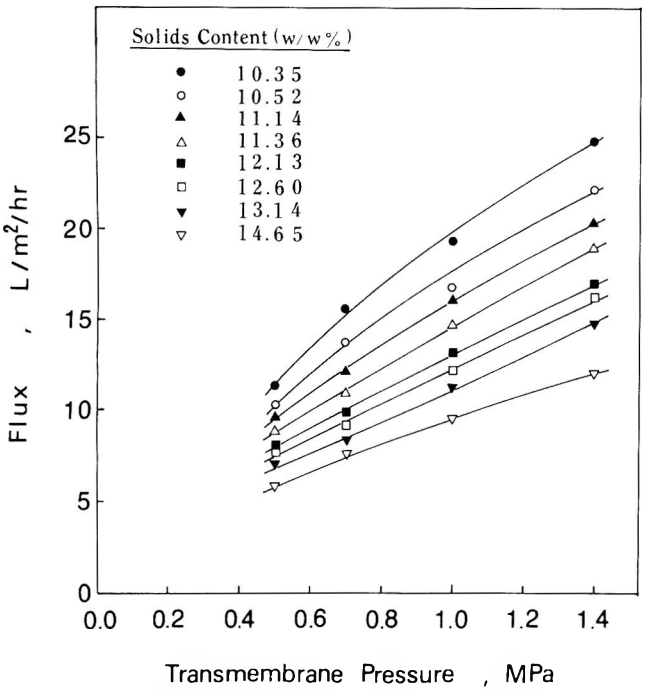

Fig. 5—Effect of transmembrane pressure and feed concentration on permeate flux, using GR81PP membrane.

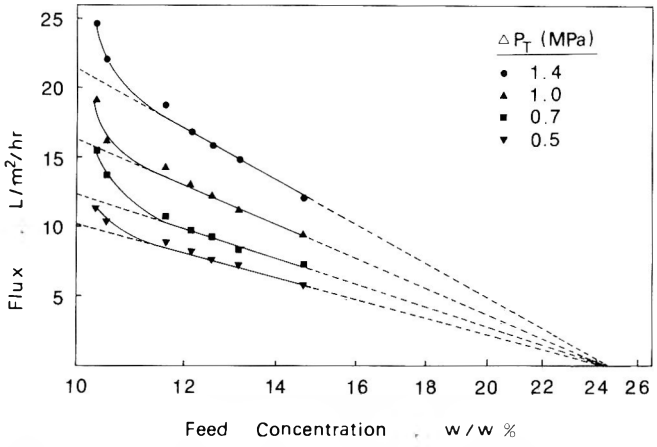

Fig. 6—Semi-log plot of permeate flux as a function of feed concentration at various transmembrane pressure, using GR81PP membrane.

above empirical mathematical model was also used to analyze the data; the results are given in Table 4. It was found that the b values did not show significant correlation with operating pressure. This indicated that the fixed flow rate of 7.3 L/min used in this study may have been too low to reveal the effect of pressure on fouling.

Figure 5 illustrates the effect of feed concentration and operating pressure on permeate flux. The data of flux (J) vs natural logarithm of feed concentration (ln C_b) is given in Fig. 6. The relationship was found linear, except at low feed concentration. It is believed that the high flux at low feed concentration was due to incomplete concentration polarization on the surface of the membrane. Extrapolating the linear portion of the graph to zero flux resulted in a single point at the x-axis of approximately 24.5% total solids. This observation suggested that the process could be expressed by gel polarization model of UF processes, $J = K \ln (C_g/C_b)$, where K is the mass transfer coefficient; C_g is the gel concentration on the membrane surface at steady state (Michaels, 1968). C_g in this study was estimated to be 24.5% total solids.

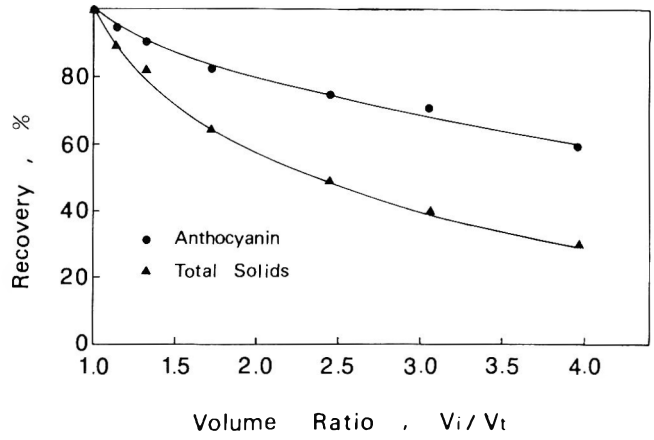

Fig. 7—Recoveries of anthocyanin and total solids as a function of anthocyanin concentration ratio, using GR81PP membrane at 1.4 MPa transmembrane pressure.

Table 5—Composition of UF permeate

Total solids	8.97 ± 0.59 %
Soluble solids	8.26 ± 0.48 %
Anthocyanin	0.004 ± 0.001%

Anthocyanin recovery

Figure 7 gives the percent recovery of anthocyanins and total solids at various volume concentration ratio (V_i/V_t). At the volume concentration of 4, the percent recovery of total solids was below 30%. The relatively low recovery of total solids makes the concentration process for anthocyanin by UF membrane possible. However, the limited effective membrane area (0.072 m^2) used in this experiment required long periods to achieve the desired concentration. The mechanical abuses by recycling the retentate may have damaged some of the pigment. The recovery ratio probably could be greatly improved by use of large scale operations. After UF concentration, the permeate still contains some free anthocyanin pigments in approximately 9% citric acid solution (Table 5). The UF permeate should be reused as extracting solvent.

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Rectified Concentrated Grape Must: HPLC Analysis of Phenolic Substances and Hydroxymethylfurfural

C. POMPEI, M. ROSSI, and E. BAROZZI

ABSTRACT

The phenolic compounds extracted with ethyl acetate from 8 grape musts (5 Italian, 2 Greek, and 1 French) and from the rectified concentrated musts (RCMs) obtained from these, were analyzed. No quantifiable amount of phenolics were found by HPLC analyses of the RCMs. However, the chromatograms showed a peak that corresponded to hydroxymethylfurfural (HMF). By direct analysis of the RCMs, under the same analytical conditions used for phenolic compounds the HMF content of the RCMs was determined to range from 26 to 99 mg per kg of product.

INTRODUCTION

RECTIFIED CONCENTRATED grape must (RCM) is a new winemaking tool which can replace sucrose in the amelioration of low-sugar musts intended for fermentation. The sugaring of must by sucrose, which is regulated within the European Community Countries, is the cause of an artificial excess of wine production which must be compensated by distillation. The use of RCM makes it possible to enrich the must with a grape product comparable in purity to sucrose without altering the sensory features of the wine. RCMs are produced by the elimination of cations, anions and phenolic compounds from the must by ion-exchange. The product leaving the ion-exchange column is a colorless liquid containing a mixture of glucose and fructose in the same proportions as in the original must, with traces of substances other than sugars. This liquid is concentrated by evaporation in vacuo until a soluble solids with a concentration between 65% and 70% is reached (Pompei, 1982; Dupuy, 1984).

Production and utilization of RCM were authorized by the ECC Commission, and Regulation 459/80 (Anonymous, 1980) establishes the specifications. Specifications state that RCMs must contain more than 100 and less than 400 mg of total phenolics per kg of sugar, of these at least 50% are to be simple phenols. These limits have been widely criticised. The above values are based on the analytical methods of Singleton and Rossi (1965) and of Kramling and Singleton (1969). However, the phosphomolybdic-phosphotungstic acid reagent recommended by these methods is not specific for phenolic substances. It has in fact been shown that sugars and SO_2 interfere with quantitative analysis of musts (Moutounet, 1981) and that the results obtained depend upon the size of sample tested (Junge, 1983). Moutounet (1982) analyzed RCM samples using the method of Singleton and Rossi (1965) both directly and after passing the sample through a Sephadex LH20 and then eluting the phenolics with acetone. In the former case the various samples contained the equivalent of 250–350 mg of phenolics per kg of sugar; in the latter case, phenolic substances were found to be absent. Clearly, in direct analysis the quantitation was affected, quite probably by the presence of reducing sugars. Therefore, another method to determine phenolic substances in samples rich in sugars such as musts and RCMs would be desirable. Results of RCM analysis based on the Folin-Ciocalteu reagent showed that the anionic resins used in producing RCMs preferentially remove high molecular weight phenols of a tannic nature rather than simple phenols; thus, these latter are expected to be found in RCMs (Razzari et al., 1978; Santini, 1982).

The aim of this research was to use high performance liquid chromatography (HPLC) to determine low molecular weight phenolics in RCMs.

Authors Pompei and Rossi are affiliated with the "Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Sezione Tecnologie Alimentari," Università di Milano, via Celoria, 2, 20133 Milano, Italy. Author Barozzi's present affiliation: Ettore Arrigoni sr., corso Lodi, 102A, 20139 Milano, Italy.

MATERIALS & METHODS

Musts

Analysis of low molecular weight phenolics was carried out on eight samples of RCMs and on the original highly sulfited grape musts (*mutés*) from the following grapes: A- white "Catarratto" grape (free-run juice), Italy; B- white "Catarratto" grape, Italy; C-aromatic white table grape "Italia," Italy; D- white "Trebbiano" grape, Italy; E- red "Uva d'oro" grape, Italy; F- red "Romeic" grape, Greece; G- red "Corinthian Black" grape, Greece; H- white grape, France.

The RCMs were obtained using the "Ampelos" process (Pompei, 1982; Dupuy, 1984) in a pilot plant situated at Strasatti (Sicily, Italy). Both the *mutés* and their respective RCM samples were stored in closed containers at about 20°C and in the dark until analysis.

Extraction of phenolic substances

To verify the reduction of phenolics following the rectification process, HPLC analysis was performed on the ethyl acetate extracts obtained from the *mutés* and on the extracts obtained from the RCMs. Extraction of low molecular weight phenols was achieved by a simplified version (Rossi and Pompei, 1986) of the method described by Villeneuve et al. (1982). The *muté* and RCM (rediluted to a sugar content equal to that of the corresponding *muté*), after the addition of 20% ammonium sulfate and 2% hydrochloric acid were extracted three times with equal volumes of ethyl acetate. The three organic fractions were combined and evaporated on a rotary evaporator (<40°C) under vacuum with nitrogen flow until the solvent was completely removed. The residue was dissolved in 10 mL of methanol and filtered through a 0.45 μm Millipore filter. The methanol was evaporated as above and the residue dissolved in 2 mL of methanol.

High-performance liquid chromatography

Analysis was carried out with an HPLC apparatus including U6K injector, 6000A pump, and 440 spectrophotometric detector (each item from Waters Assoc., Mildford, MA), connected to an Omniscribe recorder (Houston Instruments, Austin, TX).

Phenolics were separated with a reverse-phase μBondapak Phenyl column (300 mm × 3.9 mm, Waters Assoc.) fitted with a pre-column filled with μBondapak C_{18} Corasil (Waters Assoc.). Conditions were as follows: mobile phase, water/methanol/acetic acid (80:18:2, by volume); flow rate 1.5 mL/min; detector wavelength 280 nm.

Chemicals used were: acetic acid and chromatographic-grade methanol (Merck, Darmstadt, W. Germany); pure water for HPLC was obtained with demineralized water freed from organic compounds by passage over Norganic (Trace Organic Removal Cartridges, Millipore Corp., Bedford, MA) and filtration on 0.45 μm Millipore filter. A reference solution of phenolic compounds (10^{-3}M) was prepared with:

*Table 1—Composition of **mutés** (MM) and of rectified concentrated musts (RCM)[a].*

	Sample A		Sample B		Sample C		Sample D		Sample E		Sample F		Sample G	
	MM	RCM	MM	RCM	MM	RCM	MM	RCM	MM	RCM	MM	RCM	MM	RCM
pH	2.7	3.3	3.4	4.8	2.8	3.7	2.8	2.4	2.8	2.8	3.3	3.5	3.0	4.1
Relative density 20°C (g/L)	1.0852	1.3440	1.0968	1.3692	1.0791	1.3483	–	1.3309	1.0589	1.3423	1.0978	1.3525	1.1009	1.3367
Reducing sugars (g/L)	216.4	934.9	228.9	990.4	189.3	936.4	131.4	901.0	121.7	915.6	245.2	945.7	275.4	898.2
Total phenols (mg/L)[b]	576.4	138.1	936.6	192.0	860.3	225.5	893.1	330.0	2180.6	242.0	1199.6	98.5	2799.3	135.6
Simple phenols (mg/L)[c]	273.4	86.8	563.0	171.3	475.1	229.3	225.8	285.0	725.2	242.0	837.8	100.4	1812.5	143.6
Titratable acidity (meq/kg)[d]	536.0	0.7	540.0	0.3	724.0	1.3	962.0	6.9	1306.0	4.1	449.0	1.2	418.0	0.4
Total cations (meq/kg)[de]	222.0	2.6	233.0	4.3	396.0	2.5	656.0	1.0	535.0	2.7	260.0	1.4	199.0	1.8

[a] (Santini, 1982).
[b] By the method of Singleton and Rossi (1965)
[c] By the method of Kramling and Singleton (1969).
[d] Referred to reducing sugars.
[e] Calculated as differences between titratable acidity after and before treatment with an excess of cation-exchange resin (Peri et al., 1976).

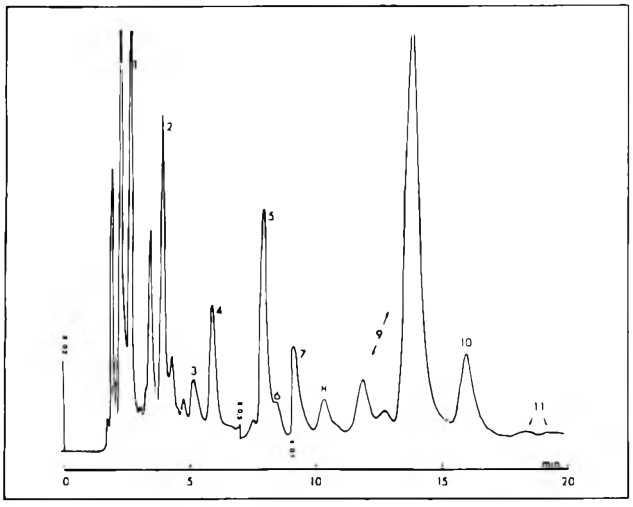

Fig. 1—HPLC chromatogram of muté extract D: column μBondapak Phenyl; mobile phase: water/methanol/ acetic acid (80/18/2, by volume); flow rate, 1.5 mL/min; wavelength, 280 nm. (1) gallic acid, (2) protocatechuic acid, (3) catechin, (4) p-hydroxybenzoic acid, (5) caffeic acid, (6) vanillic acid, (7) epicatechin, (8) syringic acid, (9) *p*-coumaric acid, (10) salicylic acid, (11) ferulic acid.

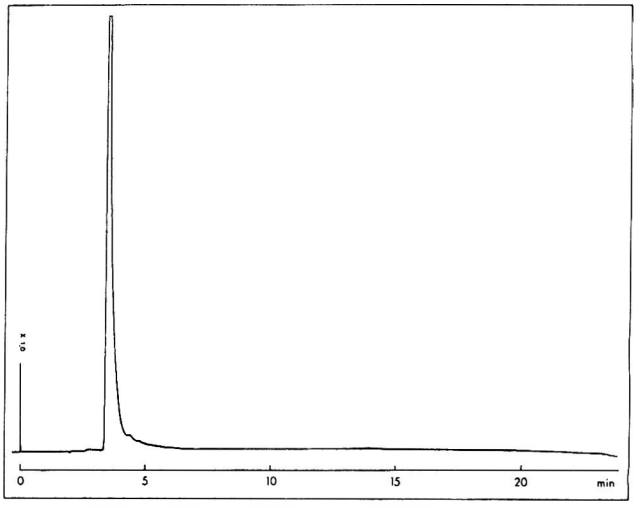

Fig. 2—HPLC chromatogram of the RCM extract C: column μBondapak Phenyl; mobile phase: water/methanol/acetic acid (80/18/2 by volume); flow rate, 1.5 mL/min; wavelength, 280 nm.

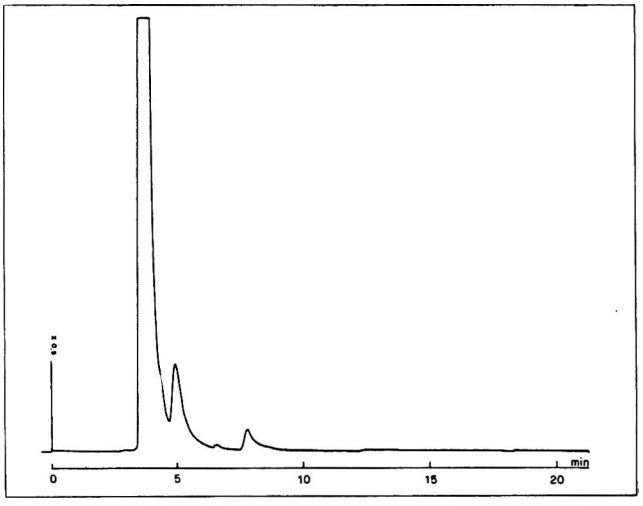

Fig. 3—HPLC chromatogram of a standard solution of hydroxymethylfurfural: column μBondapak Phenyl; mobile phase: water/methanol/acetic acid (80/18/2), by volume); flow rate, 1.5 mL/min; wavelength, 280 nm.

gallic acid (Carlo Erba, Milan, Italy); vanillic acid, p-hydroxybenzoic acid, p-coumaric acid (Ega Chemie, Stenheim, W. Germany); protocatechuic acid, salicylic acid, syringic acid (Sigma Chem. Co., St. Louis, MO); (+) catechin, (−) epicatechin, trans-ferulic acid, caffeic acid (Fluka AG, Buchs, Switzerland). A reference solution of hydroxymethylfurfural (Ega Chemie) 0.1 % v/v was also prepared.

RESULTS & DISCUSSION

THE ESSENTIAL COMPOSITION of the samples is given in Table 1 (Santini, 1982). Figure 1 gives an example of the chromatographic separation achieved with one of the *mutés* (sample D). In the analytical conditions described above, by comparison with standard substances, it was possible to recognize 13 peaks assignable to 11 phenolic compounds in the sample. Some unknown peaks probably assignable to tartaric acid esters and to their glucose esters were also observed in the chromatograms (Ong and Nagel, 1978; Singleton and Trousdale, 1983). The total phenolics extractable in ethyl acetate and identified in these musts varied from a minimum concentration of 12 mg/L (sample A) to a maximum of 36 mg/L (sample D) for white-grape musts, and from a minimum of 80 mg/L (sample F) to a maximum of 182 mg/L (sample G) for red-grape musts.

The chromatography profiles obtained from RCMs were all analogous to the one shown in Fig. 2, in which an important peak with a retention time of 3.5 min was observed. On the basis of retention time this peak corresponded to none of the reference phenolic compounds. Since no other peaks were found even at maximum signal amplification, it may be supposed that the Ampelos rectification process led to a high level of elimination of even those low molecular weight phenols which were least absorbed by the resins. This result was obtained on musts of widely varying phenolic composition; therefore, the degree of purity of the RCM obtained by this process was independent of the composition of the original must.

The comparison of the results obtained by HPLC analysis for RCMs with the values for total and simple phenolics for

Table 2—Hydroxymethylfurfural (HMF) of the RCM samples one year after production

RCM	HMF mg/L	HMF mg/kg of reducing sugars[a]
A	42.0	45.0
B	43.8	42.2
C	35.0	37.4
D	132.0	146.4
E	81.0	88.4
F	35.6	37.6
G	114.2	127.2
H	38.0	–

[a] RCMs reducing sugars are quantitated in Table 1.

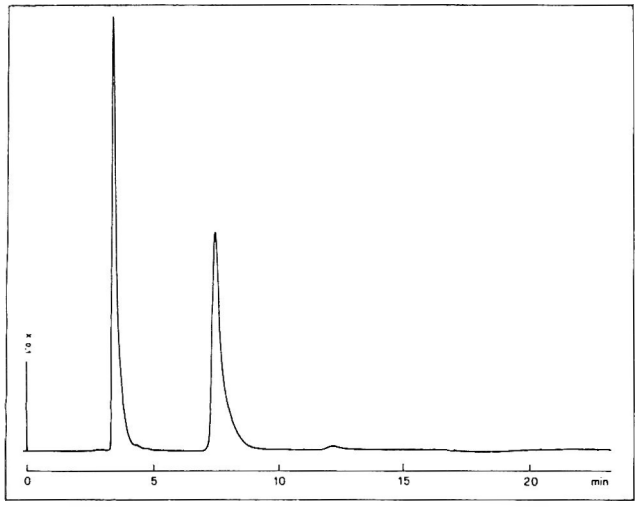

Fig 4—HPLC chromatogram of the extract obtained after two years' storage of RCM C: column μBondapak Phenyl; mobile phase: water/methanol/acetic acid (80/18/2, by volume); flow rate, 1.5 mL/min; wavelength, 280 nm.

RCMs given in Table 1 confirm the findings of Moutounet (1982) as to the impossibility of using the method of Singleton and Rossi (1965) for rectified concentrated musts.

To identify the unknown peak in the chromatogram, other compounds which absorb at 280 nm and are found in sugar-rich media were taken into consideration. One of these compounds, which has been identified in RCMs, is hydroxymethylfurfural (HMF) which, though absent in the fresh product, may reach tens of mg/L in products kept for long periods at temperatures above 25–30°C (Dupuy, 1984). HPLC analysis of a reference solution of HMF, performed according to the same method as for the phenolics, resulted in the chromatographic profile shown in Fig. 3, where a main peak with retention time 3.5 min corresponded exactly to the peak found in RCM chromatograms. The identification of this peak as HMF was confirmed by GLC-mass spectrometry.

For quantitative analysis of the HMF contained in RCM samples, it was impossible to use the same extracts which had been prepared for the analysis of phenols. It was determined, using an aqueous solution of HMF, that the method of extraction adopted for the phenols was not suitable for HMF. HPLC analysis showed that recovery from an HMF solution was very low (about 50%) and of limited reproducibility (± 20% between one extraction and another). To evaluate the quantity of HMF in the RCMs, samples diluted 1:10 (w/v) in distilled water and filtered through a 0.45 μm Millipore filter rather than RCM extracts were used. Direct injection of the diluted RCMs yielded a chromatographic profile where no peaks other than HMF as the main peak were observed, even at maximum signal amplification. Thus HMF present in the RCMs could be quantitated.

HMF concentration are given in Table 2. Values were very high, especially since the maximum limit recently established by the ECC Commission for this compound is 25 mg/kg of sugar (Anonymous, 1985). The high HMF level in the samples can be explained in two ways. Some of the *mutés* used had been obtained about a year before actually being rectified, and during this period had been stored under unknown conditions. Probably a proportion of the HMF in the samples containing the higher levels came from the original *muté*. The second explanation is that the RCMs had been stored at room temperature, which is not the ideal condition.

HPLC analysis of RCM samples two years after their production shows the presence of a second important peak at a retention time of 7.5 min (Fig. 4).. This second peak, which was not found in the earlier analysis (Fig. 2) was instead present in the reference HMF solution (Fig. 3). This peak has not yet been identified but it may be a derivative from HMF.

CONCLUSIONS

HPLC analysis showed that RCMs obtained by the Ampelos process contained no phenolic compounds and that the positive results obtained with the Folin-Ciocalteu reagent were false, referring probably to reducing sugars.

This fact should be considered by the ECC Commission so as to establish the method of determination of phenols in RCMs. HPLC may be used for simultaneous analysis both of HMF and of phenolic compounds, if both are present.

A low HMF concentration in RCMs cannot be obtained simply by controlling the conditions in which they are stored, in particular as regards temperature; the quality of the raw material must also be taken into account.

Mutés intended for the production of RCMs must be kept at suitable temperatures, which may give rise to some problems in warmer regions.

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Passion Fruit Juice Concentration by Ultrafiltration and Evaporation

ZER RAN YU and BEEN HUANG CHIANG

ABSTRACT

Passion fruit juice was pretreated with pectinase, centrifugation, and pasteurization. The resulting juice was processed by ultrafiltration (UF) to 20°Brix. The UF permeate was concentrated by evaporation to 70°Brix and combined with the UF retentate to form the final concentrated product at 40°Brix. The pretreatments caused approximately 20% flavor losses, but accomplished more than 50% flux increases. The UF recovered many important flavor constituents, and improved the rate of evaporation. The final concentrated product contained more than 30% of juice flavor. Results of sensory evaluation indicated that flavor of the reconstituted juice was inferior to fresh juice, but the two juices were not significantly different in overall acceptance.

INTRODUCTION

PASSION FRUIT (genus passiflora), a tropical fruit, has long been recognized for its unique attractive flavor. It is usually consumed in the form of juice. The distinct aroma also makes the passion fruit juice a desirable ingredient for many formulated beverages and food products. The compositions and processing procedures of passion fruit juice are covered extensively elsewhere (Casimir et al., 1981; Chan, 1980; Luh, 1980; Pruthi, 1963).

Concentration of passion fruit juice could be done using vacuum evaporator. However, recovery of the volatile flavoring constituents during evaporation was essential to avoid flavor deficiency (Casimir et al., 1981). Membrane concentration technology has been used to concentrate a number of fruit juices (Merson and Morgan, 1968; Matsuura et al., 1974; Dale et al., 1982; Sheu and Wiley, 1983; Wilson and Burns, 1983; Kirk et al., 1983) due to its low energy requirement (Pepper, 1980; Parkinson, 1983). Pompei and Rho (1974) used cellulose acetate membranes on a plate-and-frame reverse osmosis (RO) module to concentrate passion fruit juice from 16.8 to 28% solids, and found a 41.6 to 69.1% retention for aroma constituents. However, the authors indicated that the concentration ratio was limited by the allowable maximum operating pressure of the RO limit.

Concentration of fruit juices by membranes was generally done by RO. Ultrafiltration was commonly considered inadequate for fruit juice concentration for its low retention ability for aroma compounds due to high porosity of the membrane. However, in a previous study it was found that many important flavor compounds in the passion fruit juice could be retained by UF membrane, possibly due to their association with macromolecules (Yu et al., 1986). The objective of this study was to further investigate the possibility of using UF, connected with an evaporator, to concentrate passion fruit juice.

MATERIALS & METHODS

Passion fruit juice preparation

Extraction of passion fruit juice from hybrid variety of *Passiflora edulis* Sims. (female) and *Passiflora edulis* var. flavicarpa (male) was done with a brush finisher equipped with 1.0 and 0.5 mm screens (Keeseng Machine Co., Tainan, Taiwan). The resulting juice was then screened through 80 mesh and 200 mesh screeners to remove broken seeds and fibers.

The authors are with the Graduate Institute of Food Science and Technology, National Taiwan Univ., Taipei, Taiwan, ROC. Address inquiries to Dr. Chiang.

Pretreatments

The pretreatments for the juice process included enzyme treatment to hydrolyze pectic substances, centrifugation to remove starch, and pasteurization to kill microorganisms and inactivate enzymes. The enzyme treatment was conducted at 30°C for 1 hr using 100 ppm pectinase (Ultrazym 100, Swiss Ferment Co., Ltd., Switzerland). A disc-type centrifugal separator (Model SA-1-02-135, Westfalia, W. Germany) operated at 9000 rpm (4950g), 1 bar of outlet pressure and feed rate 1 L/min, was used for centrifugation. The pasteurization process was conducted at 75°C for 40 sec using plate heat exchanger (Model UHX, Iwai Industrial Co., Japan).

Ultrafiltration process

The PCI Laboratory RO/UF unit (Paterson Candy International Ltd., Witchurch, England) equipped with B1 tubular module was used for UF. The main reason for choosing a tubular system was its ability to concentrate fluids of high viscosity without permanent fouling (Paulson et al., 1984). The PCI BX6 UF membrane with a nominal molecular weight cut-off of 25,000 daltons, was found to retain many important passion fruit aroma compounds in a previous study (Yu et al., 1986) and was used in this study. This membrane can be used at pH 2–12 up to 70°C with typical operating pressure of 3–15 bar.

Factors affecting the performance of UF include operation pressure, feed concentration, temperature and feed velocity. Since the volatile compounds of passion fruit juice are heat sensitive, the temperature for the UF operation was 20 ± 2°C. The feed velocity was fixed at 4.1 m/sec. To study the effects of pretreatment on UF, the feed juice was tested for permeation rate at constant pressure (13 bar) for 2 hr. Permeation rates were also studied at various feed concentrations and operation pressures. The testing pressures ranged from 10 to 14 bar. The feeds at different concentrations were expressed as weight concentration ratio (WCR), which was the initial batch weight of the feed divided by the weight of the retentate after the permeate was removed by UF. During the studies, both permeate and retentate were recycled to the feed tank to maintain a steady operation condition.

Evaporation process

A rotary vacuum evaporator (Model N1, Tokyo Rikakikai Co., Ltd. Japan) equipped with aspirator (Model A-2S) was used for evaporation. The process conditions were at 25 mmHg absolute pressure, 45 ± 1°C, and 6 rpm rotating speed. Five hundred ml solution was concentrated. Samples were taken at 0, 20, 40, and 60 min after processing, and analyzed for concentration of soluble solids to determine the rate of evaporation.

Analytical

The total solids were determined according to AOAC (1980) method. The pH was measured at 20°C using a pH meter (Basic, Digital pH/mv meter, Chin Chi Co., Ltd., Taiwan). The viscosities of the solutions at 20°C were determined using Brookfield synchro-lectric viscometer (Model LVT, Brookfield Engineering Laboratories, Ltd. MA). The Brookfield LV NO. 1 or NO. 2 spindles were used, depending on the fluid viscosity. Changes in color of the juice during processing were determined with a color difference meter (Model C-5220, Tokyo Denshoku Co., Ltd. Japan), and expressed as L, a, b values (Pomeranz and Meloan, 1978). The starch in the juice was determined by a colorimetric method (Kwok et al., 1974). Absorbance was measured at 565 nm, which corresponded to the maximum absorption, using a spectrophotometer (Shimadzu Double Bean Spectrophotometer, Model UV-200S, Japan). The D-galacturonic acid content in the sample so-

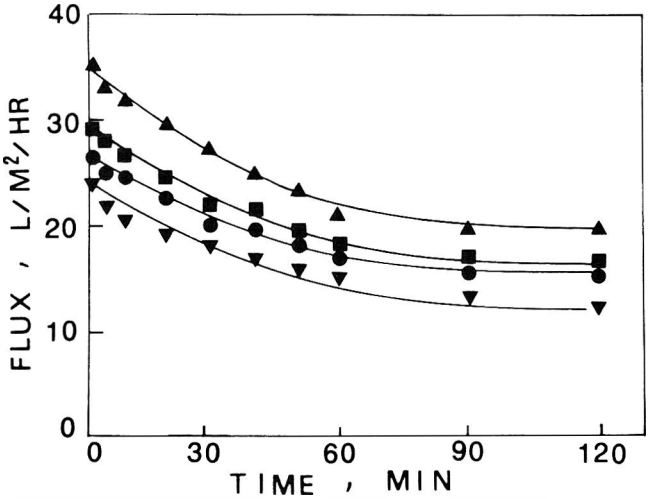

Fig. 1—Effects of various pretreatments on premeation rates of ultrafiltration of passion fruit juice using PCI tubular UF system equipped with BX6 UF membrane. ▼, untreated juice; ●, juice after enzyme treatment; ■, juice after centrifugation; ▲, juice after enzyme treatment plus centrifugation.

Table 1—Effects of pretreatments on physicochemical properties of passion fruit juice

Component	Treatments			
	Control	Centrifugation	Enzyme	Enzyme plus centrifugation
Total solids (%)	18.3[a]	16.6[bd]	18.2[a]	16.5[cd]
pH	2.9[a]	2.9[a]	2.9[a]	2.9[a]
Viscosity (cps)	30.5[a]	11.1[b]	23.5[c]	9.1[d]
Color				
L	48.0[a]	32.5[bd]	46.0[a]	32.3[cd]
a	6.8[a]	1.4[b]	4.8[c]	1.2[b]
b	31.1[a]	19.1[bd]	28.8[a]	18.1[cd]
Total pectin (%)	0.22[a]	0.19[b]	0.15[c]	0.13[d]
Acids (%)				
citric acid	4.09[a]	4.09[a]	4.05[a]	4.03[a]
L-malic acid	0.43[a]	0.43[a]	0.42[a]	0.43[a]
latic acid	0.70[a]	0.70[a]	0.65[a]	0.65[a]
Sugars (%)				
fructose	3.43[a]	3.41[a]	3.30[a]	3.31[a]
glucose	3.53[a]	3.56[a]	3.41[a]	3.43[a]
sucrose	5.02[a]	4.92[a]	5.11[a]	5.09[a]
Flavor				
Total volatiles (ppm)	969.4[a]	808.1[b]	848.8[c]	761.0[d]
Total flavor profile	5323.3[a]	4485.7[bd]	5163.4[a]	4437.4[cd]

[a-d] Means within rows followed by the same letter are not significantly different at the 5% level.

lution was determined by the carbazole method (Bitter and Muir, 1962). Assuming that pectin contained 77% D-galacturonic acid (Luh, 1980), the pectin content of the juice was estimated.

Sugars were analyzed by high performance liquid chromatograph (HPLC) (Waters Assoc., Milford, MA) using a Lichrosorb NH_2 column (E. Merck Co., Germany) with acetonitrile/H_2O (75/25, v/v) as eluting solvent. Organic acids were separated on a Lichrosorb RP-18 column (E. Merck Co., Germany) using potassium dihydrogen phosphate and phosphoric acid buffer solution at pH 2.4 as eluting solvent.

Volatile compounds in the sample solution were swept onto a Tenax-GC adsorption polymer (Supelco Inc., Bellefont, PA) with nitrogen gas, using the headspace method of Chen et al. (1982). The adsorbed volatiles were desorbed and collected on a 3 m × 3 mm glass column packed with 3% Carbowax 20 M on 80/100 mesh Chromosorb G/HP (E. Merck Co., Germany) and analyzed in a Gasukuro Kogyo Model 370 gas chromatograph (Japan) equipped with a FID detector. Total flavor volatiles in the sample was determined by summation of the peak areas from the GC chromatogram using SIC 7000AS integrator (System Instruments Co., LTD. Tokyo, Japan), and the concentration (expressed as ppm) was estimated by comparing the total peak area with that of the internal standard (ethyl cinnamate). Changes of flavor quality due to processing were also evaluated based on total flavor

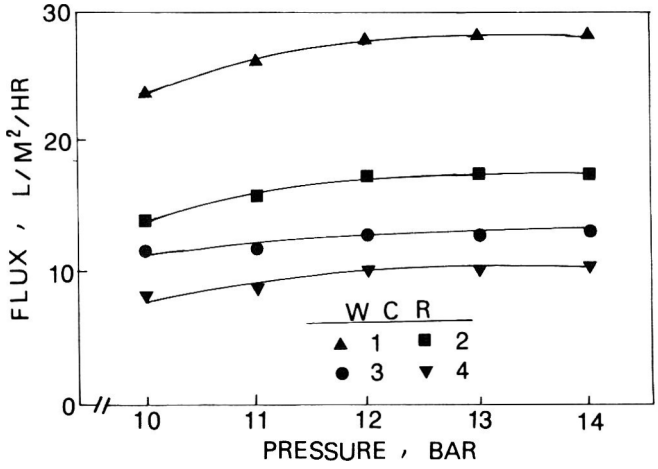

Fig. 2—Relationships between permeate flux and operation pressures during UF concentration processes of passion fruit juice at various WCR. WCR = initial wt of feed/final wt of retentate.

Table 2—Effect of pasteurization (75°C, 40 sec) on the physical and chemical properties of passion fruit juice

Component	Before pasteurization	After pasteurization
Total solids (%)	15.2[a]	14.9[a]
pH	2.9[a]	2.9[a]
Viscosity (cps)	7.4[a]	7.4[a]
Color		
L	32.2[a]	32.2[a]
a	1.2[a]	−0.3[b]
b	17.9[a]	17.1[a]
Acids (%)		
citric acid	3.70[a]	3.68[a]
L-malic acid	0.43[a]	0.40[a]
lactic acid	0.61[a]	0.59[a]
Sugars (%)		
fructose	3.00[a]	2.99[a]
glucose	3.07[a]	3.09[a]
sucrose	5.03[a]	4.99[a]
Flavor		
total volatiles (ppm)	775.9[a]	412.4[b]
total flavor profile	5457.5[a]	3932.8[b]

[a,b] Means within rows followed by the same letter are not significantly different at the 5% level.

profile (TFP), which was calculated based on the formula; TFP = Σ (Flavor impact value × concentration), as described by Casimir and coworkers (1981).

The analytical determinations were carried out in duplicate. The data were analyzed statistically by the test of least significant difference (LSD) (Steel and Torrie, 1960).

Sensory evaluation

The fresh juice and the juice concentrate were diluted to 4 °Brix (ca. ¼ concentration of single strength passion fruit juice). Sucrose were then added to the juices until the concentration reached 12 °Brix. The prepared juices at 10 ± 2°C were evaluated by 18 experienced panelists for color, flavor, taste and overall acceptance based on a hedonic scale, where 9 was like extremely; 1 was dislike extremely (Larmond, 1982). The panelists were chosen from the graduate students of this institute, all of them had been involved in sensory evaluations periodically for various products. During sensory tests, the panelists were requested to evaluate four unidentified juice samples, 100 mL each cup, in an open area supplied with sufficient light by light bulbs. The four samples were two fresh juices and two reconstituted juices. The data obtained from sensory evaluations were then analyzed statistically by analysis of variance.

RESULTS & DISCUSSION

Effect of pretreatments

The purpose of pretreatment was to increase permeate flux of membrane processes. The single strength passion fruit juice

Table 3—Changes of physicochemical properties of the permeates and retentates at various WCR during ultrafiltration process

Component	Single strength juice	Permeate			Retentate		
		WCR = 2[d]	WCR = 3	WCR = 4	WCR = 2	WCR = 3	WCR = 4
Total solids (%)	14.9	13.0[a]	13.7[b]	14.1[c]	17.3[a]	18.5[b]	19.7[c]
pH	2.9	2.9[a]	2.9[a]	2.9[a]	2.9[a]	2.9[a]	2.9[a]
Viscosity (cps)	7.4	3.5[a]	3.5[a]	3.8[b]	13.5[a]	22.0[b]	35.0[c]
Color							
L	32.2	84.5[a]	84.9[a]	85.8[b]	37.4[a]	38.0[ac]	39.4[bc]
a	−0.3	−1.8[a]	−1.9[a]	−2.1[a]	2.5[a]	4.1[b]	5.3[c]
b	17.1	8.8[a]	9.4[ac]	9.8[bc]	21.14[a]	22.6[b]	23.7[b]
Acids (%)							
citric acid	3.68	3.41[a]	3.51[b]	3.56[b]	3.91[a]	4.03[b]	4.13[b]
L-malic acid	0.40	0.36[a]	0.36[a]	0.36[a]	0.40[a]	0.41[b]	0.42[b]
lactic acid	0.59	0.51[a]	0.53[ac]	0.54[bc]	0.62[a]	0.66[b]	0.69[c]
Sugars (%)							
fructose	3.02	2.56[a]	2.56[ac]	2.71[bc]	2.89[a]	3.07[b]	3.18[c]
glucose	3.13	2.55[a]	2.70[b]	2.83[c]	2.94[a]	3.16[b]	3.27[c]
sucrose	4.83	3.63[a]	4.02[b]	4.70[c]	5.57[a]	6.37[b]	6.85[c]
Flavor compounds (ppm)							
ethyl acetate	50.0	19.9[a]	23.5[a]	18.3[a]	21.7[a]	26.2[a]	22.0[a]
methyl butyrate	5.9	1.5[a]	2.0[a]	1.6[a]	3.2[a]	4.2[b]	4.7[c]
ethyl butyrate	147.7	50.1[a]	52.6[b]	46.4[a]	62.5[a]	65.0[a]	56.5[a]
ethyl hexanoate	18.1	2.2[a]	2.2[a]	2.0[a]	17.3[a]	15.2[a]	15.2[a]
2-heptanol	10.1	1.4[a]	1.4[a]	1.4[a]	5.9[a]	6.1[a]	5.8[a]
1-hexanol	6.4	3.7[a]	4.2[ac]	4.2[bc]	6.6[a]	7.4[a]	7.8[a]
cis-3-hexenol	2.3	0.0[a]	0.0[a]	0.0[a]	1.2[a]	1.4[a]	1.7[a]
hexyl butyrate	41.1	0.4[a]	0.4[a]	0.6[b]	51.7[a]	86.8[b]	114.6[c]
cis-3-hexyl butyrate	7.2	0.1[a]	0.0[a]	0.0[a]	8.1[a]	12.1[b]	15.7[c]
ethyl cis 3-octenoate	2.4	0.0[a]	0.0[a]	0.0[a]	2.8[a]	4.2[b]	5.5[c]
ethyl-4, 7-octadienoate	1.6	0.6[a]	0.6[a]	0.6[a]	1.2[a]	1.0[a]	1.4[a]
linalool	1.5	0.4[a]	0.4[a]	0.4[a]	1.3[a]	1.5[a]	2.4[b]
hexyl hexanoate	22.2	0.0[a]	0.0[a]	0.0[a]	29.0[a]	51.2[b]	68.9[c]
β-ionone	3.2	1.4[a]	1.8[a]	1.8[a]	4.1[a]	7.7[b]	9.5[c]
Total volatiles	412.4	106.2[a]	114.1[b]	91.2[c]	315.7[a]	456.0[b]	542.5[c]
Total flavor profile	3932.8	747.8[a]	899.9[b]	826.8[c]	5080.6[a]	8033.4[b]	10223.4[c]

[a-c] Means within rows of permeate and retentate followed by the same letter are not significantly different at the 5% level.
[d] WCR: weight concentration ratio = initial weight of the feed/final weight of UF retentate.

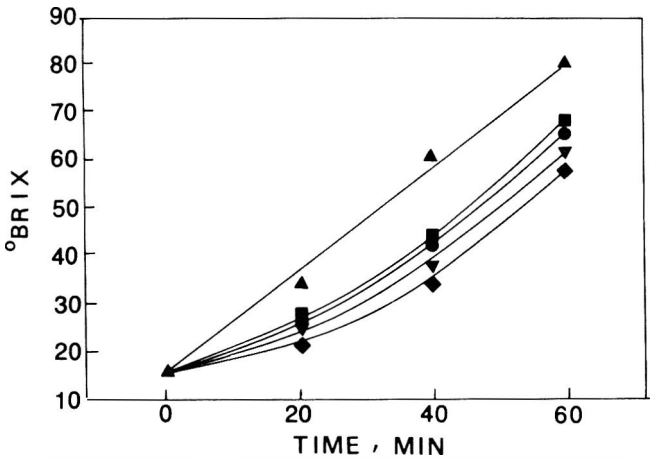

Fig. 3—Evaporation rates of various products. ♦, untreated juice; ▼, enzyme treated juice; ●, centrifuged juice; ■, enzyme treated and centrifuged juice; ▲, UF permeate.

contained approximately 0.22 w/v% total pectin and 2.2 w/w % starch. They should be removed to avoid later processing difficulties. Preliminary tests showed that 100 ppm pectinase (Ultrazym 100, optimal pH 4.0–4.5, Swiss Ferment Co., Switzerland) was only able to hydrolyze approximately 30% of total pectin in 1 hr at 30°C. Further increase in the enzyme concentration or incubation time did not reduce the pectin content, possibly due to limited enzyme activity at low pH (2.9) of the juice. The processing conditions, 9500 rpm (4950g); 1 bar outlet pressure; 1L/min feed rate, for centrifugal operation were determined based on machine capacity and previous experience with the separator. Under these conditions nearly 90% of starch could be removed from the juice, which is comparable to that found by Casimir (1974) using a decanting centrifuge. The effects of enzyme treatment and centrifugal operation on the physico-chemical properties of the juice are given in Table 1. Their effects on permeate flux of UF are shown in Fig. 1. As expected, the centrifugal operation was detrimental to flavor constituents of the juice as a result of the intimate contact between the juice and air during spinning process as shown in Table 1. A similar finding was also reported by Kuo and coworkers (1985). Although the pretreatments caused approximately 20% flavor loss, it increased the flux more than 50%.

The major sugars in the passion fruit juice are fructose, glucose and sucrose (Chan and Kwok., 1975). Citric acid, L-malic acid and lactic acid are the major organic acid (Chiou, 1984; Chan et al., 1972). The pretreatments did not affect the contents of acids and sugars. Centrifugation removed starch in the juice, thus reduced the total solids slightly, but decreased the viscosity of the juice significantly. Centrifugation also reduced the pectin content in the juice. Enzyme treatment, however, was most effective in pectin reduction. Due to the reduction of pectic substances, the viscosity of the juice was decreased significantly by enzyme treatment. Centrifugation also reduced the color intensity of the juice. The pigments may be associated with the starch, fibers and other suspended solids and were lost during desludging process of centrifugation.

The de-pectinized and de-starched juice was also pasteurized at 75°C for 40 sec before concentration. Pasteurization caused approximately 45% flavor losses based on total volatiles (Table 2). However, if the changes of flavor quality was estimated by TFP, only 28% of flavor loss was due to pasteurization. Other physicochemical properties of the juice were not altered by pasteurization, except the intensity of red color of the juice, which decreased a little, possibly due to the destruction of carotenoid in the juice by heat.

The pasteurization conditions used in this study were based

Table 4—Comparisons of physicochemical properties of fresh and reconstituted passion fruit juices

Component	Fresh juice	Reconstituted juice
Total solids (%)	14.9[a]	14.8[a]
pH	2.9[a]	2.9[a]
Viscosity (cps)	7.4[a]	7.4[a]
Color		
L	32.2[a]	31.4[a]
a	−0.3[a]	−0.9[b]
b	17.1[a]	16.7[a]
Acids (%)		
citric acid	3.68[a]	3.66[a]
L-malic acid	0.40[a]	0.39[a]
lactic acid	0.59[a]	0.58[a]
Sugars		
fructose	2.99[a]	3.04[a]
glucose	3.09[a]	3.09[a]
sucrose	4.99[a]	5.01[a]
Flavor compounds (ppm)		
ethyl acetate	50.0[a]	6.2[b]
methyl butyrate	5.9[a]	0.7[b]
ethyl butyrate	147.7[a]	14.8[b]
ethyl hexanoate	18.1[a]	3.8[b]
2-heptanol	10.1[a]	1.8[b]
1-hexanol	6.4[a]	2.5[b]
cis-3-hexenol	2.3[a]	0.1[b]
hexyl butyrate	41.1[a]	31.6[b]
cis-3-hexyl butyrate	7.7[a]	5.4[b]
ethyl cis-3-octenoate	2.4[a]	1.8[b]
ethyl-4,7-octadienoate	1.6[a]	0.2[b]
linalool	1.5[a]	0.3[b]
hexyl hexanoate	22.2[a]	21.4[a]
β-ionone	3.2[a]	0.6[b]
Total volatiles	412.4[a]	136.5[b]
Total flavor profile	3932.8[a]	2293.8[b]

[a,b] Means within rows followed by the same letter are not significantly different at the 5% level.

on Chiou's (1984) report that for long term frozen storage the passion fruit juice should be pasteurized at 75°C for 40 sec. Many researchers indicated that thermal processing of passion fruit juice would cause considerable flavor losses (Kuo et al., 1985; Aung and Ross, 1965; Kefford and Vicker, 1961; Seale and Sherman, 1960), which was also found in this study. Since the flavor quality changes estimated by TFP were much smaller than that estimated by total volatiles, it appeared that the volatile compounds, which have high flavor impact value (FIV), were more heat stable. Since high FIV flavor compounds may have more imput flavor quality of the juice, the TFP might be a better indicator for evaluating the effect of pasteurization on flavor of the juice.

Ultrafiltration of passion fruit juice

The relationships between operation pressures and permeate flux at various WCR are shown in Fig. 2. Increases in pressure increased the flux slightly. When the pressure exceeded 12 bar, a further increase could not improve flux. The pressure independency at high operation pressure may be attributed to the phenomenon of concentration polarization (Michaels, 1968). The flux decreased as the WCR increased. An equation, $J = K_1 - K_2 \ln WCR$ where J is the permeate flux in L/hr·m^2, WCR is the weight concentration ratio, and K_1 and K_2 are constants) similar to that formulated by Breslau and Kilcullen (1977) was used to analyze the experimental data. It appeared that the data fitted this model well (correlation coefficient = 0.99). K_1 and K_2 were found to be 26.4 and 12.3 L/hr·m^2, respectively.

The changes in physicochemical properties of the permeates and retentates during UF are shown in Table 3. As expected, the effect of concentration by UF was small. However, since most of the macromolecules were retained by UF membrance, the viscosity of the retentate increased rapidly as the concentration ratio increased. Increased viscosity would reduce the turbulency of the fluid flow in the tubes of the UF system, which was probably partially responsible for the low permeation rate at high WCR. UF process did not affect pH significantly because of its low retentivity for acids. Recovery of sugars were also low. Most of the pigments of the juice were retained in the retentate. The intensity of the color increased as the WCR increased.

The effect of concentration by UF for the volatile compounds in the juice was relatively high, compared to sugars and acids. A possible explanation was that the flavor compounds were associated with macromolecules, which were retainable by UF membrane. The major flavor compounds which had high retentivities by the UF membrane were hexyl butyrate, cis-3-hexyl butyrate, hexyl hexanoate, β-ionone and linalool. Due to their high FIV, the concentration effect for flavor estimated by TFP was particularly high.

Evaporation process

After UF, the permeate contained sugars, acids and some flavor components. The possibler foulants for evaporation, such as pectin and other macromolecules, were retained in the retentate. Therefore, the problems of foaming and fouling could be reduced when concentrating the UF permeate by evaporation. The differences in evaporation rates for various feed so-

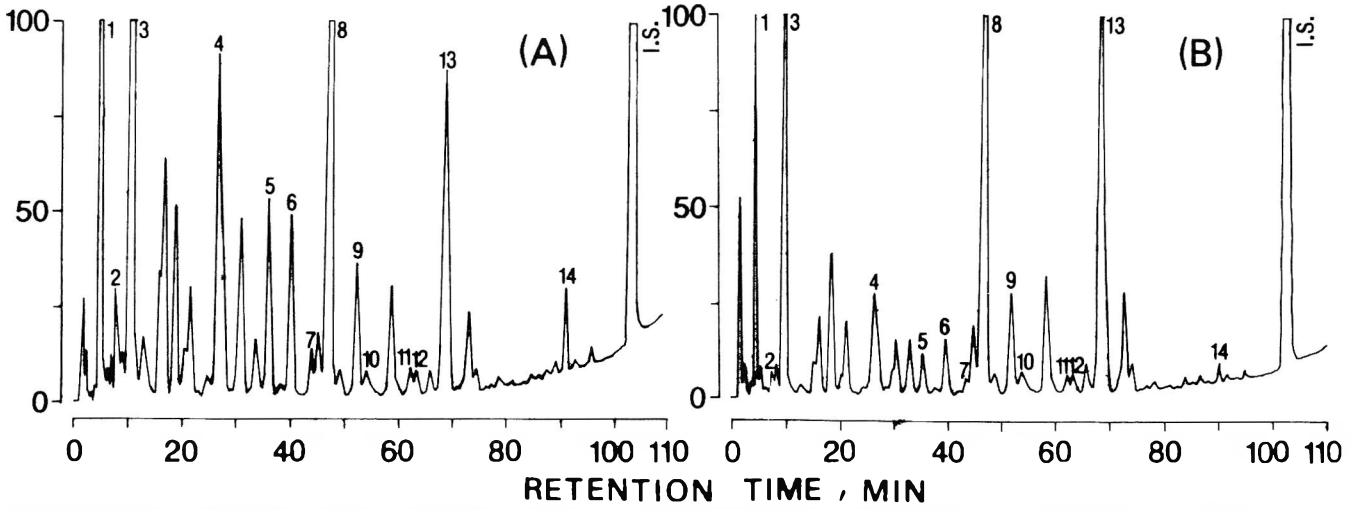

Fig. 4—Gas chromatograms of passion fruit juice volatile compounds. (A) the fresh juice; (B) the reconstituted juice, (1) ethyl acetate; (2) methyl butyrate; (3) ethyl butyrate; (4) ethyl hexanoate; (5) 2-heptanol; (6) 1-hexanol; (7) cis-3-hexenol; (8) hexyl butyrate; (9) cis-3-hexyl butyrate; (10) ethyl cis-3-octenoate; (11) ethyl-4,7-octadienoate; (12) linalool; (13) hexyl hexanoate; (14) β-ionone; I.S. = internal standard, ethyl cinnamate.

lutions are given in Fig. 3. If the solutions are to be concentrated to 60 °Brix, 40 min is required for UF permeate. The solutions without UF treatment would require 50 to 60 min. This result demonstrated that the rate of evaporation could be increased by UF. Nearly 90% of volatile components in the UF permeate were lost during evaporation. The other components and the physical properties of the UF permeate were not changed, except that the color became more yellowish.

Evaluation of product quality

The UF retentate at WCR 4 (~ 20 °Brix) was combined with the concentrated UF permneate (~ 70 °Brix) to yield the final juice concentrate (~ 40 °Brix). After diluting the concentrated product with distilled water, the reconstituted juice was evaluated for physicochemical properties, compared with the single strength juice (Table 4). It was found that the properties of the UF/evaporation product were nearly identical to that of the fresh juice, except for the concentration of the flavor compounds. The GC profiles for the fresh and reconstituted juice are given in Fig. 4. The losses of volatile components, estimated by total GC peaks area was approximately 70%. If the aroma intensity was estimated by TFP, only 42% of flavor compound loss was found. The high percent recovery of TFP was attributed to the good retention of many high FIV compounds in the juice.

The reconstituted and original juices were evaluated by sensory panels. The color and taste of the two samples were not significantly different ($P > 0.05$). The flavor of the original juice was superior to the reconstituted juice ($P < 0.05$; the mean scores were 6.9 and 6.0 respectively). However, based on overall acceptance, the two samples were not significantly different ($P > 0.05$; the mean scores were 6.0 and 6.3 for reconstituted juice and fresh juice, respectively).

CONCLUSIONS

ULTRAFILTRATION (UF) in this study could be considered as a prefiltration step. It removed macromolecules from the passion fruit juice so that the rate of evaporation could be increased due to better efficiency of heat transfer. UF also served as a fractionation process. Along with the retained macromolecules, many important flavor compounds were kept in the UF retentate. When the UF retentate was added back to the concentrated UF permeate, the flavor quality of the juice was largely restored.

Enzyme treatment and centrifugation process prior to UF were necessary to increase the permeation rate. The thermal processing condition, 75°C for 40 sec, resulted in a significant flavor compound loss. It appeared that a better process condition should be sought to minimize the flavor damage during heat treatment.

This study presents an possible means of utilizing UF in passion fruit juice concentration. However, future research is still needed for improving both the flavor compounds retention ability and the permeation rate of UF in order to assure the successful application of UF in the process.

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Extraordinary Heat Resistance of *Talaromyces flavus* and *Neosartorya fischeri* Ascospores in Fruit Products

L. R. BEUCHAT

ABSTRACT

Ascospores of three strains each of *Talaromyces flavus, Neosartorya fischeri* and *Byssochlamys fulva/nivea* were analyzed for resistance to thermal inactivation in five fruit-based (blueberry, cherry, peach, raspberry and strawberry) products. $D_{91°C}$ values for two strains of *T. flavus* ranged from 2.9–5.4 min; $D_{88°C}$ values ranged from 7.1–22.3 min. Ascospores of *N. fischeri* were somewhat less heat resistant; $D_{91°C}$ values were < 2.0 min and $D_{88°C}$ were 4.2–16.2 min. Ascospores of *Byssochlamys* spp. were considerably less heat resistant. The type of fruit product did not appear to substantially influence rates of thermal inactivation. No heat-resistant ascospores of *T. flavus* or *N. fischeri*, i.e., ascospores capable of surviving 15 min at 75°C, were formed on fruit products stored at 10°C for 137 days. However, *T. flavus* and *N. fischeri* formed ascospores on cherry substrate stored at 25°C within 65 and 137 days, respectively, that survived 15 min at 88°C.

INTRODUCTION

ASCOSPORES of *Byssochlamys fulva* and *B nivea* (anamorphic stages = *Paecilomyces fulvus* and *P. niveus*, respectively), *Talaromyces flavus* (= *Penicillium dangeardii* [Pitt = *Penicillium vermiculatum* Dangeard]) and *Neosartorya fischeri* (= *Aspergillus fischeri*) are extremely heat resistant compared to those of other molds and have been reported to cause spoilage of heat-processed fruits and fruit products (Beuchat and Rice, 1979; Hatcher et al., 1979; Hocking and Pitt, 1984; Jesenska et al., 1984; Kavanagh et al., 1963; McEvoy and Stuart, 1970). Once formed, ascospores may persist in a dormant state for months or even years in decaying fruit debris and soil, and consequently can be isolated from fruits harvested from or close to the ground and from containers and equipment used to transport and process fruit. These ascospores are able to survive heat pasteurization treatments routinely applied to most fruits and fruit products (e.g., 90°C for 3 min) and, thus, spoilage may occur due to post-pasteurization germination of ascospores and subsequent outgrowth (Beuchat and Rice, 1979; Splittstoesser and Splittstoeser, 1977). Some strains of *Byssochlamys* are capable of producing patulin, a mycotoxin of considerable potency (Rice et al., 1977; Roland and Beuchat, 1984).

Byssochlamys spp. are historically among the most widely encountered molds causing spoilage of heat-processed fruits and have, therefore, been most extensively researched. Recently, however, spoilage due to *T. flavus* and *N. fischeri* has been more frequently observed in North America, Europe, and Australia. The organisms can be found on a variety of fruits at the time of harvest (Hocking and Pitt, 1984). It has been reported that *Talaromyces* and *Neosartorya* ascospores can surive 100°C for 5 to 12 min (Kavanagh et al., 1963; McEvoy and Stuart, 1970; Van der Spuy et al., 1975). Environmental factors such as pH, oxygen tension, water activity and the presence of certain organic acids in fruits as well as strain variation can influence rates of heat inactivation of vegetative cells and ascospores of yeasts and molds. The influence of these parameters on heat inactivation of *Byssochlamys, Talaromyces* and *Neosartorya* ascospores has not been thoroughly investigated.

The study reported here was designed to determine the effects of time, temperature and type of fruit used as a suspending medium on rates of inactivation of three strains each of *T. flavus, N. fischeri* and *B. fulva/nivea*. The production of heat-resistant ascospores on fruit products stored at 10° and 25°C was also investigated.

Author Beuchat is with the Dept. of Food Science, Univ. of Georgia Agricultural Experiment Station, Experiment, GA 30212-5099.

MATERIALS AND METHODS

Organisms

Three strains each of *T. flavus* and *N. fischeri*, two strains of *B. nivea* and one strain of *B. fulva* were selected for the investigation (Table 1).

Procedure for producing and harvesting ascospores

Oatmeal wheat germ agar (OWA) was used to culture the test molds. This medium consisted of (g per liter of tap water): oatmeal, 15g; wheat germ, 15 g; and agar, 20g. The mixture was boiled for 30 min and filtered through six layers of cheesecloth; the filtrate was sterilized by autoclaving for 15 min at 121°C. The final pH (unadjusted) was 6.46. The medium was poured into Petri dishes and allowed to "dry" at 21°C for 1–2 days.

Mold propagules from active cultures of the nine test molds were suspended in 0.1M potassium phosphate buffer (buffer, pH 7.0) and surface plated (0.1 mL) on the OWA medium. Cultures were incubated at 30°C. Ages of *Byssochlamys* spp., *T. flavus* and *N. fischeri* cultures were 49, 57, and 64 days, respectively, when ascospores were harvested.

To harvest ascospores, the surface of cultures was flooded with buffer and gently rubbed with a sterile bent glass rod. The suspension was filtered through sterile glass wool to remove hyphal fragments and then sonicated at 0–4°C to disrupt the asci, thereby freeing ascospores into the buffer. Sonication time varied, depending upon the strain under investigation. Ascospore suspensions were stored at 4°C until tested for heat resistance.

Preparation of fruit products

Commercial fruit fillings consisting of fruit (blueberry, cherry, peach, raspberry and strawberry), sugar, water, locust bean gum, pectin and calcium chloride were used as media in which to suspend ascospores during heat treatment. Products were homogenized aseptically in a high-speed Waring Blendor and deposited (100 mL) in sterile 250-mL Erlenmeyer flasks. The flasks were immersed up to the neck in an oil bath (recycling) adjusted to temperatures ranging from 70°C (158.0°F) to 91°C (195.8°F), depending upon the strain under study. Magnetic stirring bars were used to constantly mix the fruit puree during the heating period.

Procedure for determining temperature and time necessary for activation

The temperature and time necessary for activation of ascospores was determined. Buffer (100 mL in 250-mL Erlenmeyer flask) adjusted to 70, 75, 80 or 85°C was inoculated with 1.0 mL of ascospore suspension ($A_{600\ nm}$ = 0.56 – 0.59). Samples were withdrawn from the continuously agitated suspension at 15-min intervals over a 120-min period, serially diluted in buffer and surface-plated (0.1 mL) on plate count agar containing rose bengal (25 μg/mL) and chloramphenicol (100 μg/mL) (PCRBC, pH 7.0). Plates were incubated at

Table 1—Strains and sources of molds examined for thermal inactivation characteristics in fruit products

Mold	Strain	Source
T. flavus	NFPA-2	National Food Processors Assoc., Washington, DC
	CBS 317-63	Centralbureau voor Schimmelcultures, Baarn, The Netherlands
	FRR-1265	Commonwealth Scientific and Industrial Research Organization, North Ryde, Australia (CSIRO)
N. fischeri	FRR-2334	CSIRO
	FRR-1833	CSIRO
	110483	Laboratory stock
B. nivea	NRRL-2615	Northern Regional Research Lab., USDA, Peoria, IL
	ATCC-28221	American Type Culture Collection, Rockville, MD.
B. fulva	A-22921A	Laboratory stock

30°C for 4 days before colonies were counted and colony-forming units (cfu) per mL of buffer were calculated.

Procedure for determining thermal resistance in fruit products

Thermal inactivation characteristics (D and z values) of each test mold were investigated. One milliliter of ascospore suspension ($A_{600\ nm}$ = 0.56 – 0.59) was deposited and immediately dispersed in the fruit puree (100 mL in 250-mL Erlenmeyer flask) which was adjusted to the desired temperature in an oil bath. After selected time periods ranging to 120 min, samples (5 mL) were withdrawn from the heating mixture, deposited in sterile test tubes immersed in ice, serially diluted in phosphate buffer and surface-plated (0.1 mL) on PCRBC agar. Plates were incubated at 30°C for a minimum of 5 days before mold colonies were counted. Decimal reduction times over the linear portion (at least three log cycles) of inactivation curves and z values for each test strain heated at various temperatures were calculated.

Shelf life study

Equal volumes of ascospore suspensions ($A_{600\ nm}$ = 0.55) of *T. flavus* NFPA-2 and CBS 317-63 were combined and diluted 10^{-2} in buffer. Fruit purees (250 mL) adjusted to 85°C were inoculated with 2.5 mL of the diluted suspension, constantly mixed over a 30-min period and deposited (20–22g) in sterile Petri dishes. Quadruplicate samples were wrapped in plastic bags, incubated at 10° and 25°C, and visually examined daily for growth of *T. flavus*.

Outgrowth of heat-activated ascospores of *N. fischeri* in fruit purees stored at 10° and 25°C was likewise studied using the same procedure described for *T. flavus*. A mixture of approximately equal populations of *N. fischeri* strains FRR-2334, FRR-1833 and 110483 was used as an inoculum.

The presence of heat-resistant ascospores formed by *T. flavus* and *N. fischeri* which eventually grew on fruit purees incubated at 10 and 25°C was determined. The entire puree sample (20-22 g) was combined with 80 ml of sterile buffer (pH 7.0) and homogenized in a Stomacher for 2 min. Ten milliliters of the homogenate were transferred to 90 mL of buffer tempered at 75° and 88°C in a 250-mL Erlenmeyer flask. After 15 min of constant agitation, a 4-mL sample was withdrawn, deposited in a chilled test tube, serially diluted in buffer and surface-plated (0.1 mL) on PCRBC. Colonies were counted after 6 days of incubation at 30°C.

RESULTS & DISCUSSION

PRIOR TO INVESTIGATING thermal inactivation characteristics of mold ascospores, the pH and soluble solids (as measured using a refractometer) of fruit products were determined (Table 2). The pH ranged from 3.19 (strawberry) to 3.87 (cherry) with soluble solids from 25.2% (cherry) to 33.4% (raspberry).

Table 2—pH and soluble solids of fruit products

Fruit product	pH	Soluble solids (%, 21°C)
Blueberry	3.46	27.8
Cherry	3.87	25.2
Peach	3.31	29.8
Raspberry	3.19	30.3
Strawberry	3.47	33.4

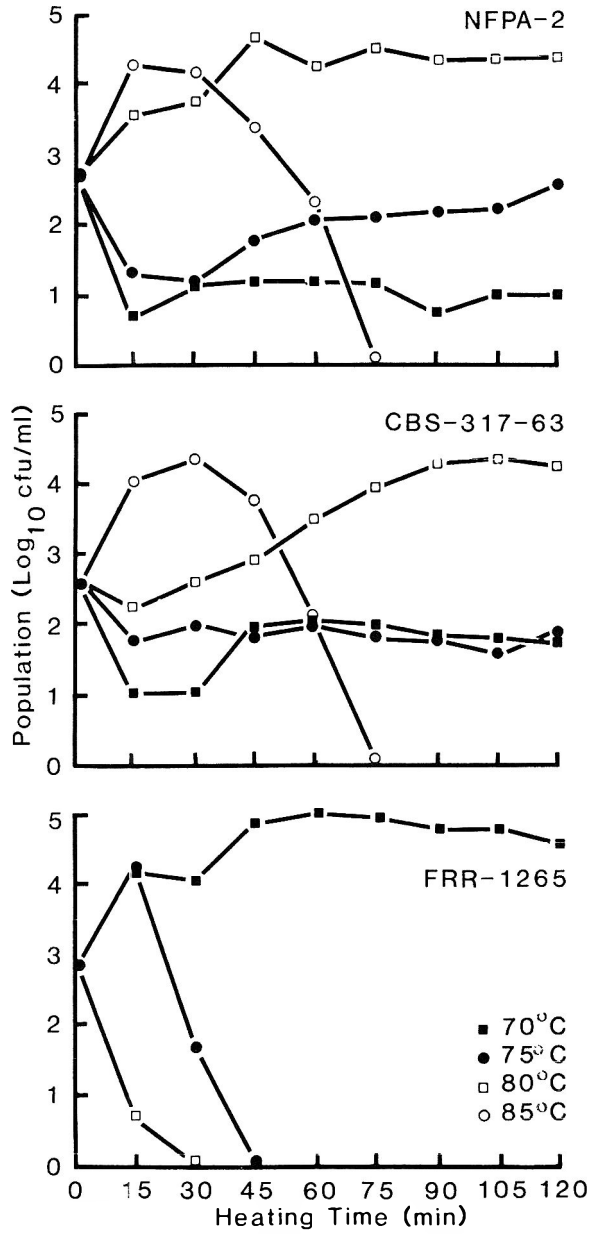

Fig. 1—Thermal activation/inactivation curves for three strains of Talaromyces flavus ascospores heated at 70, 75, 80 and 85°C in 0.1M potassium phosphate buffer (pH 7.0).

Temperature and time necessary for activation

The effects of heating ascospores of *T. flavus* at 70°, 75°, 80°, and 85°C in buffer for periods of time ranging to 120 min are illustrated in Fig. 1. Strains NFPA-2 and CBS 317-63 exhibited similar heat resistant characteristics whereas strain FRR-1265 was somewhat less heat resistant. Strain FRR-1265 formed smaller ascospores than the other two strains, and this may be correlated with relative heat resistance.

Several notable observations should be made from data presented in Fig. 1. At 70° and 75°C, a very large percentage (> 99%) of the ascospores of strains NFPA-2 and CBS 317-63 was not heat activated to the extent that they would germinate and form colonies on PCRBC agar. This was true even when ascospores were heated for as long as 120 min. The optimum

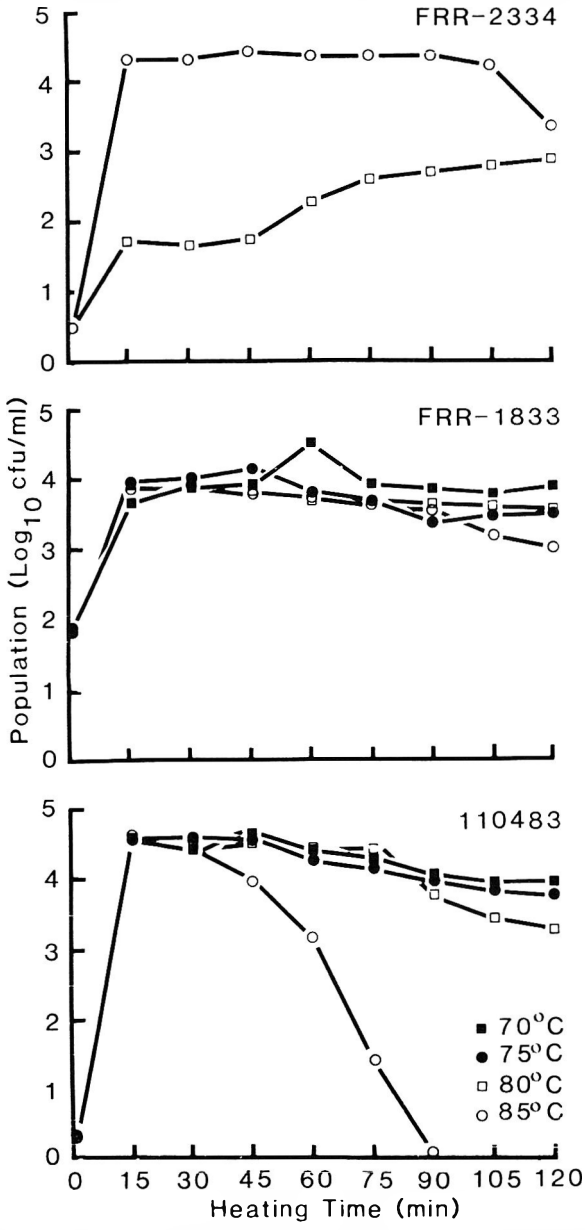

Fig. 2—Thermal activation/inactivation curves for three strains of Neosartorya fischeri ascospores heated at 70, 75, 80, and 85°C in 0.1M potassium phosphate buffer (pH 7.0).

temperatures tested for activating these two strains was 85°C for 15–30 min or 80°C for ≥ 45–120 min and ≥ 90–120 min, respectively, for strains NFPA-2 and CBS 317-63. Heating times in excess of 30 min at 85°C resulted in inactivation of ascospores. Any decreases in detectable populations of strains NFPA-2 and CBS 317-63 during the first 15 min of heating were probably due to inactivation of hyphal fragments and conidia present in the inoculum and not to inactivation of ascospores.

The optimum temperature for activating strain FRR-1265 was 70°C. Full activation was reached in about 45 min followed by a slow rate of inactivation through 120 min; total inactivation within 15 and 30 min occurred at 85° and 80°C, respectively.

Thermal activation/inactivation curves at 70°, 75°, 80°, and 85°C for three strains of *N. fischeri* are shown in Fig. 2. Ascospores of strain FRR-2334 required the highest temperatures for activation; maximum activation occurred only at 85°C. Heating at 70° and 75°C failed to activate ascospores of this strain.

Strain FRR-1833 behaved somewhat differently in that all test temperatures (70°, 75°, 80°, and 85°C) activated ascospores in a similar fashion. Likewise, ascospores of strain 110483 were activated similarly at all temperatures; however, treatment at 85° for ≥ 45 min caused a rapid decline in viable ascospores. Ascospores heated at 70°, 75°, and 80°C for longer than about 45 min lost viability, but at a much slower rate than at 85°C.

Ascospores of *B. nivea* and *B. fulva* exhibited considerably less heat resistance than those of *T. flavus* and *N. fischeri*. Activation was achieved within 30 min at 70° and 75°C followed by complete inactivation at 90 min. At 85°C, ascospores did not survive 15-min treatments. The relative heat resistance of the *Byssochlamys* species examined in this study would therefore not be expected to pose the serious inactivation problem associated with *T. flavus* and *N. fischeri*. Other strains of *Byssochlamys*, however, would easily withstand 15 min at 85°C (Beuchat and Rice, 1979).

There are some very practical implications which can be drawn from data illustrated in Fig. 1 and 2. The heat processing scheme used to manufacture some types of fruit products may result in activation of dormant mold ascospores. Data presented here are from a very limited number of strains but nevertheless demonstrate a rather wide range of time/temperature combinations necessary for heat activation. This range encompasses processing schema necessary to achieve desired sensory characteristics while also inactivating microorganisms with less heat resistance. Thus, heat processing may also guarantee activation of ascospores of some strains of molds.

Data illustrated in Fig. 1 and 2 should also be considered when raw or processed samples are analyzed for total yeast and mold populations. Heat-resistant mold ascospores may not be detected if such samples are not subjected to heat treatment before plating on an appropriate nutrient medium. This is best illustrated by our inability to detect *N. fischeri* FRR-2334 ascospores that were not heated at 80° and 85°C for at least 15 min (Fig. 2). While the population of heat-resistant ascospores in raw fruits would be expected to be low (perhaps < 1/100 g) or absent, it may be necessary to subject samples to heat treatment to facilitate detection. Thus, using traditional methodology, a detected population of 10 (yeasts plus molds) per 50g may not reflect the presence of ascospores which require heat shock to induce activation followed by colony formation.

Data in Fig. 1 and 2 were derived from tests in which buffer (pH 7.0) was used as the heating medium. These data cannot be directly extrapolated to fruit products since rates of activation/inactivation of ascospores are likely to be influenced by the nature of the heating medium (Splittstoesser and Splittstoesser, 1977). However, similar trends could be predicted in fruit products. Ascospores may be protected by solutes such as sugar in the products but, at the same time, acid pH may enhance rates of inactivation.

Thermal resistance in fruit products

The pH and soluble solids of fruit products are listed in Table 2. The range of these characteristics is similar to that of other fruit-based formulations used in the food industry, and thus results of studies reported here may have application in a broader sense.

Listed in Table 3 are D values for three strains of *T. flavus* and *N. fischeri* heated in five fruit products. Measurable D values (> 2.0 min) for *Byssochlamys* spp. using our experimental design could be determined only at temperatures below about 170°F (77°C). The relative heat resistance of these strains compared to *T. flavus* and *N. fischeri* was low, thus making information on their D and z values less significant in the overall objective to determine survival characteristics of mold ascospores at time/temperature conditions given to most fruit products. Extensive investigations to determine D and z values of *Byssochlamys* at temperatures less than 170°F (77°C) were, therefore, discontinued.

Table 3—D values of Talaromyces flavus and *Neosartorya fischeri* in five fruit products

Mold	Strain	Temp °F (°C)	D value[a], (min) in: Blueberry	Cherry	Peach	Raspberry	Strawberry
T. flavus	NFPA-2	179.6 (82)	> 120	> 120	> 120	> 120	> 120
		185.0 (85)	68.1	26.5	28.5	26.2	52.0
		190.4 (88)	19.2	9.3	9.7	7.1	14.3
		195.8 (91)	4.7	4.1	3.9	2.9	3.9
	CBS 317-63	179.6 (82)	> 120	> 120	> 120	> 120	> 120
		185.0 (85)	64.2	20.4	26.9	28.8	47.1
		190.4 (88)	22.3	11.2	12.0	9.1	3.5
		195.8 (91)	5.4	4.9	4.1	3.4	11.7
	FRR-1265	158.0 (70)	> 120	> 120	> 120	> 120	> 120
		163.4 (73)	9.7	9.2	11.3	8.9	9.4
		168.8 (76)	4.7	5.8	3.8	3.8	4.1
		174.2 (79)	2.5	3.5	2.2	2.1	2.2
N. fischeri	FRR-2334	179.6 (82)	> 120	> 120	> 120	> 120	> 120
		185.0 (85)	116.0	51.8	43.9	41.1	45.0
		187.7 (86.5)	53.1	19.0	20.1	16.7	15.9
		190.4 (88)	16.2	7.8	7.7	8.8	5.3
		195.8 (91)	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
	FRR-1833	179.6 (82)	> 120	> 120	> 120	> 120	> 120
		185.0 (85)	47.0	41.2	36.9	30.1	41.4
		187.7 (86.5)	22.0	15.1	17.2	15.9	19.3
		190.4 (88)	12.1	8.9	7.1	8.1	11.2
		195.8 (91)	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
	110483	179.6 (82)	> 120	> 120	> 120	> 120	> 120
		185.0 (85)	17.6	19.2	15.1	16.4	19.4
		187.7 (86.5)	9.9	7.9	9.4	10.0	10.3
		190.4 (88)	6.6	4.2	5.2	6.3	4.4
		195.8 (91)	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0

[a] D values were calculated from the linear portion of thermal inactivation curves which transgressed at least two log cycles.

T. flavus NFPA-2 and CBS 317-63 were more heat-resistant than was strain FRR-1265, regardless of the type of fruit in which heat treatment was administered. This confirms the general ranking of heat resistance observed for ascospores heated in buffer (Fig. 1). D values for strains NFPA-2 and CBS 317-63 at 195.8°F (91°C) ranged from 2.9–5.4 min; at 190.4°F (88°C), values ranged from 7.1–22.3 min. *N. fischeri* ascospores were somewhat less heat resistant than ascospores of *T. flavus*. D values at 195.8°F (91°C) were < 2.0 min; D values ranged from 4.2–16.2 min at 190.4°F (88°C), depending upon the type of fruit.

The rate of inactivation of *T. flavus* and *N. fischeri* ascospores may have been slowest in the blueberry product, although a clear case for drawing such a conclusion cannot be made. There appeared to be no correlation between the pH or soluble solids of various fruit products and rates of thermal inactivation of ascospores.

The z values for various strains of *T. flavus* and *N. fischeri* are listed in Table 4. *T. flavus* FRR-1265, in addition to being the most heat sensitive, had the largest z values (14.1–23.3°F) of the three test strains of *T. flavus*. *N. fischeri* 110483, which was slightly less heat resistant than strains FRR-2334 and FRR-1833, also had the largest z values (9.0–11.0°F). There may be some correlation between increased heat resistance and decreased z values for ascospores of *T. flavus* and *N. fischeri*. Within a particular strain, z values differed depending upon the type of fruit in which ascospores were suspended during heating. There did not appear to be a correlation between magnitude of z value and type of fruit in which ascospores were heated when considering all strains within *T. flavus* and within *N. fischeri*.

Shelf life study

The populations of heat-activated ascospores of *T. flavus* and *N. fischeri* were calculated to be 114 and 74 per gram of fruits, respectively, at the time fruits were placed into storage. Colonies of both mold species were evident on all fruits stored at 25°C for 3 days. Visible colonies of *T. flavus* appeared on all fruits between 28 and 32 days of incubation at 10°C. Fruits containing *N. fischeri* and stored at 10°C showed visible mold growth after 65 days.

No heat-resistant *T. flavus* or *N. fischeri* ascospores, i.e., ascospores capable of surviving 15 min at 75°C, were detected on fruit products stored at 10°C for 137 days, the maximum storage time evaluated. Only the cherry product supported production of heat-resistant ascospores when fruit products were stored at 25°C. Populations of $> 10^4$ ascospores of *T. flavus* per gram were detected in heated (15 min at 88°C) cherry product incubated at 25°C for 65 days. *N. fischeri* ascospores in cherry product remained viable ($> 10^4$/g) upon treatment for 15 min at 75°C but not at 88°C after 29 days of incubation at 25°C; $> 10^5$ ascospores of *N. fischeri* per gram of cherry product stored for 137 days survived in 15 min at 88°C.

Observations on fruits stored at 10°C indicate that ascospores in original inocula had either died, reverted to a state of dormancy which required a more rigorous time/temperature treatment to induce activation necessary for eventual colony

Table 4—z Values for Talaromyces flavus and *Neosartorya fischeri* in five fruit products

Mold	Strain	z value, °F Blueberry	Cherry	Peach	Raspberry	Strawberry
T. flavus	NFPA-2	9.7	13.9	12.5	10.3	9.6
	CBS 317-63	10.6	17.2	12.6	11.7	9.4
	FRR-1265	16.6	23.3	14.1	16.9	16.5
N. fischeri	FRR-2334	5.4	5.9	6.8	7.1	5.7
	FRR-1833	9.0	7.3	7.1	9.2	8.8
	100483	11.0	9.5	10.8	9.6	9.0

formation on the enumeration medium or germinated and formed colonies without subsequent production of new ascospores.

CONCLUSIONS

CONSIDERING the sensory and nutritional qualities desired in fruit products, it may not be feasible to render these products free of viable heat-resistant ascospores, if indeed they are present in raw fruit, using many current formulation and processing schemes. However, there are several approaches which might be taken to eliminate or at least reduce spoilage of fruit products by heat-resistant molds. The most obvious way would be to eliminate these molds in raw fruits as they are received from the supplier. This would be extremely difficult to do, since contamination probably occurs from soil and debris in the field before and during harvesting as well as during transport and processing at the suppliers' plants.

The addition of low concentrations of potassium sorbate and/or potassium benzoate to fruit-based formulations would be expected to lengthen shelf life. These acids would most likely enhance the rate of thermal inactivation of ascospores as well as retard outgrowth of any ascospores that may survive processing.

Refrigeration temperatures, perhaps even less than 10°C, would extend shelf life. The temperature selected would depend on the shelf life desired, the availability and refrigeration capacity of facilities and the amount of increased cost due to increased energy consumption, among other factors.

Application of vacuum to containers in which fruit products are deposited would reduce the rate of mold development. Flushing the head space of containers with nitrogen would further enhance this effect. An increase in carbon dioxide in the atmosphere at the surface of the fruit products or in the products themselves would be anticipated to reduce the rate of growth of molds in general.

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Detection of Enocyanin in Cranberry Juice Cocktail by HPLC Anthocyanin Profile

M.L. HALE, F.J. FRANCIS, AND I.S. FAGERSON

ABSTRACT

Adulteration of cranberry juice products by enocyanin, a colorant from grapes, was detected by comparing HPLC chromatographic profiles of cranberry anthocyanins with those from enocyanin. Two peaks present in all enocyanin samples but not in cranberries were identified as delphinidin-3-glucoside and petunidin-3-glucoside. A group of unidentified pigments which eluted after the cranberry pigments was also present in enocyanin. It was possible by considering differences in the chromatograms to detect replacement of 5% or more of the expected amount of cranberry juice in cranberry juice cocktail by a solution of enocyanin. A CG-50 column was used to concentrate the pigments followed by chromatography on a polymer styrene column at pH 1.6. The method gives reliable results even on samples 18 months of age.

INTRODUCTION

CRANBERRY JUICE is a relatively expensive ingredient and usually comprises about 25% of cranberry juice cocktail. Consequently, there is considerable economic incentive to reduce the amount of cranberry juice and augmennt the product with citric acid as a source of acidity and enocyanin as a colorant. Such adulteration can be detected by conventional profile analysis such as the determination of nonvolatile acids and anthocyanidins (Hong and Wrolstad, 1986a). The same authors (1986b) also provided a more complete profile of cranberry components. The ratio of citric acid to other acids such as quinic and malic normally found in cranberries may be useful as an indication of the degree of adulteration (Coppola et al., 1978). A simple color measurement was proposed as a screening test for gross adulteration (50% or more) by Francis (1985). The yellow flavonoid and red anthocyanin pigment profile as determined by chemical methods could be used to detect replacement of 25–50% of the cranberry juice (Francis, 1985). The work presented here reports an HPLC anthocyanin profile method which can be used to detect replacement of 5% of cranberry juice with an enocyanin solution.

MATERIALS & METHODS

Samples

The cranberries (*Vaccinium macrocarpon*, cultivar Early Black) used in this study were obtained from the University of Massachusetts Cranberry Experiment Station in East Wareham, MA. They were stored at −20°C until use. Eight different enocyanin samples, from American, Italian, and South African producers, were supplied by Minot Food Packers (Bridgeton, NJ). Commercial cranberry juice beverages were purchased locally.

Pigment preparation

Approximately 100g whole frozen berries was blended with 200 mL 0.1N HCl in methanol and refrigerated overnight. This macerated mixture was filtered and run through an Amberlite CG-50 ion exchange column according to the method of Fuleki and Francis, (1968). Some of this pigment solution was evaporated to dryness in an air stream and saved for HPLC use. The rest was concentrated, and the anthocyanins separated and identified by paper chromatography according to the method of Fuleki and Francis (1967). These separated pigments were used as standards for the HPLC analysis.

Authors Francis and Fagerson are with the Dept. of Food Science & Nutrition, Univ. of Massachusetts, Amherst, MA 01003. Author Hale's present address is General Foods Corp., Cranberry, NJ.

HPLC apparatus

A laboratory Data Control Constametric II G liquid chromatograph equipped with a Rheodyne Model F120, 20 ul sample loop injector, a Gradient Master Model 1601 (Milton Roy Co.), and a Spectromoniter II UV-VIS spectrophotometer Model 1202 (Milton Roy Co.) was used. The recorder, an Omni-Scribe Series B-500 (Houston Instruments), was set at a chart speed of 0.5 cm/min. Cranberry anthocyanins (in a solution of 0.1N HCl in H_2O) were separated with a 150 × 4.6 mm. i.d. column packed with 5 micron polymeric reversed phase styrene divinylbenzene column (PLRP-S) (Polymer Laboratories, Inc). A five cm guard column packed with the same material preceded the analytical column. Two mobile phases were used: solvent A contained acetic acid:water (10:90) and solvent B contained methanol:acetic acid:water (60:10:30). Both A and B also contained 3% phosphoric acid (added as a buffer). All solvents were HPLC grade and were ultrasonically degassed. Both samples and solvents were filtered through a 0.45 micron filter membrane before use.

HPLC analysis of cranberry anthocyanins

For the gradient elution analysis of anthocyanins, a flow rate of 0.8 mL was used with a concave curve (m = 3) gradient. The mobile phase ran from 0% B to 90% B in 30 min where it remained until the last peak eluted and a stable baseline was resumed. Detection was set at 530 nm.

The four major cranberry anthocyanins, which had been separated and identified by paper chromatographic methods, were individually injected into the HPLC and their retention times noted. Cranberry pigment extract, prepared as previously described, was injected into the HPLC and the elution profile similarly noted.

Analysis of enocyanin

Commercial enocyanin samples were diluted with water and purified through an Amberlite CG-50 column in the same manner as the cranberry pigment. Each sample was injected into the HPLC under the same conditions as were used for the cranberry anthocyanins. Three peaks, which appeared in every enocyanin sample, were manually collected by running one enocyanin sample a total of 17 times and collecting the eluant as it discharged from the detector (this task was made easier by the fact that the eluant turned pink as a peak eluted). These individual anthocyanins were concentrated and the aglycone was separated from the sugar by hydrolyzing with 2N HCl in a boiling water bath for 30 min followed by separation with amyl alcohol. The aglycone (upper layer) was removed, concentrated, streaked on Whatman No. 3 paper, and run for 72 hr in Forrestal solution, according to the method of Francis (1982). The three aglycones were identified using standards prepared in the same manner from blue grapes.

The position of attachment of the sugar(s) to the aglycone was determined by comparing the absorption at 440 nm to that at the maximum visible absorption. Anthocyanins with sugar substitution only at the 3-position have approximately twice the absorption of pigments substituted at the 3,5- or 5-position (Harborne, 1958).

In order to determine the minimum amount of enocyanin which could be added to a cranberry sample and still be detected by HPLC, a solution of 0.6% citric acid and 0.2% enocyanin was made up and portions were added to a commercial sample of cranberry juice cock-

tail in varying amounts. This solution of enocyanin was adopted because it had the same approximate tristimulus Hunter L value as commercial cranberry juice cocktail. These "adulterated" samples were prepared in the same manner as all others and injected into the HPLC instrument.

The pigment profile of the enocyanin chosen for the above solution is shown in Fig. 2D. This sample was the oldest and presumable the most degraded of the samples available to us and thus should constitute the most severe challenge.

RESULTS & DISCUSSION

A TYPICAL CHROMATOGRAM for cranberry anthocyanins is shown in Fig 1. The identity of each peak was confirmed by chromatography with authentic pigments obtained by paper chromatography of cranberry pigments.

The pH of the mobile phase was maintained at 1.6 by the addition of 3% phosphoric acid. At this pH, the anthocyanins were nearly entirely in their flavylium cation (red) form, and produced sharp HPLC peaks. Earlier researchers have usually worked with anthocyanins using a mobile phase with a pH greater than two since at lower pH values their silica-based columns were in danger of degradation. However Wulf and Nagel (1978) and Bronnum-Hansen and Hansen (1983) and others, found no loss in column efficiency after several months of use if the column was washed after each run. At pH values greater than two, anthocyanins exist in both the flavylium cation form and the carbinol base (colorless) form, and HPLC peaks are broader. This problem was circumvented here by the use of a polymer column, which could separate compounds at any pH without danger of degradation.

HPLC analysis of eight commercial enocyanin samples indicates that they have similar but not identical anthocyanin profiles. Six enocyanin chromatograms are shown in Fig. 2 A to F.

Three enocyanin peaks, labelled A, B, C, were selected for further analysis because: (1) they appeared in all eight enocyanin chromatograms; (2) they were usually the predominant peaks in the sample; and (3) none of these peaks corresponded to any of the cranberry anthocyanin peaks. The aglycone portions of the anthocyanins attributed to these peaks were identified by comparison with authentic standards obtained from blue grapes. Peaks A, B and C contained delphinidin, petunidin, and cyanidin respectively.

To date, the only sugar found in grape pigments is glucose (Singleton and Esau, 1969); it was therefore assumed that glucose was the only sugar present in these three enocyanin pigments. Table 1 lists the average absorbance obtained from three trials at the indicated wavelengths and HPLC retention times. The data indicate that pigments A, B and C are all monoglycosides with substitution at the 3 position. For compound C, the aglycone and spectral data indicate cyanidin-3-monoglucoside (Cn-3-G), yet the HPLC retention time is longer than for authentic Cn-3-G obtained from blackberries (Table 1). Compound C must be an isomer or derivative of Cn-3-G, or possibly a different sugar is present.

The presence of peaks A and B in a cranberry sample is indicative of added enocyanin. Peak A is probably the easiest to find since it is well separated from the cranberry peaks. A number of peaks occur after peak 5 for cranberries and these are also indicative of added enocyanin. Peak 2 for Cn-3-G occurs in very small concentration in cranberry juice and higher proportions in enocyanin. This compound is the most common anthocyanin occurring in nature and is readily obtainable. It may be a useful indicator for addition of enocyanin and also for determining retention times when other HPLC systems are used.

Figure 1 C is a chromatogram for commercial cranberry juice cocktail contaiing 10% of the solution containing 0.2% enocyanin. The small peak (A) for delphinidin-3-glucoside (Dp-3-G) is evident, but peaks B and C are masked by the cranberry anthocyanins. The peak eluting before A is not Dp-3-G but is

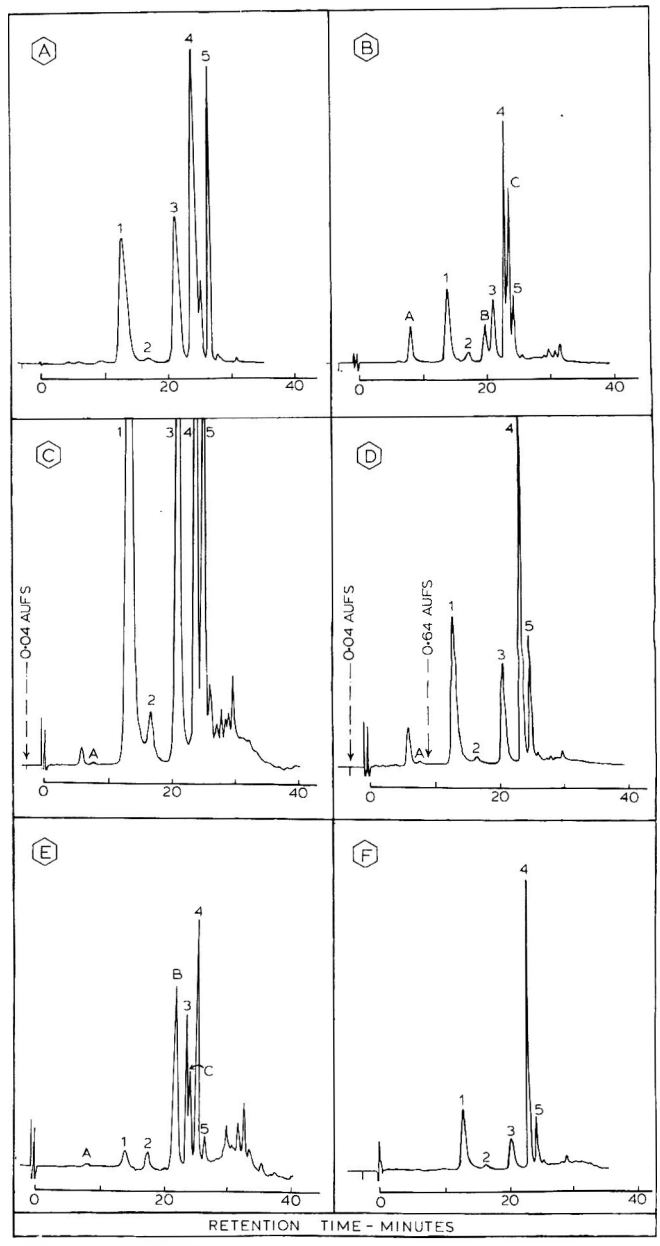

Fig. 1—(A) Anthocyanin profile from fresh cranberries. (B) Profile from a commercial sample of apple-cranberry juice with enocyanin declared on the label. (C) A commercial sample of cranberry juice cocktail with 10% of a solution containing 0.2% enocyanin and 0.6% citric acid. (D) A commercial sample of cranberry juice cocktail with 5% of a solution containing 0.2% enocyanin and 0.6% citric acid. (E) A commercial sample of cranberry juice cocktail which was considered to be adulterated by a previous method. This sample had been opened, reclosed and stored at 45°F for 18 months. (F) A commercial sample of cranberry juice cocktail which had been opened, reclosed and stored at 45°F for 7 months. Absorbance 0.64 AUFS (absorption units full scale) unless otherwise indicated. The peaks 1,2,3,4, and 5 refer to cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside, peonidin-3-galactoside, and peonidin-3-arabinoside, respectively. The peaks A, B, and C refer to delphinidin-3-glucoside, petunidin-3-glucoside, and cyanidin-3-glucoside, respectively.

an unidentified minor pigment coming from the cranberry component, as evidenced from an increase in size with increased cranberry juice content. It is only evident at the very high HPLC sensitivity (0.04 absorption units full scale) indicated in Fig. 1 C and D, thus it must be present in extremely low concentrations in cranberry juice. Fig. 1 D is a chromatogram for commercial cranberry juice containing 5% of the solution

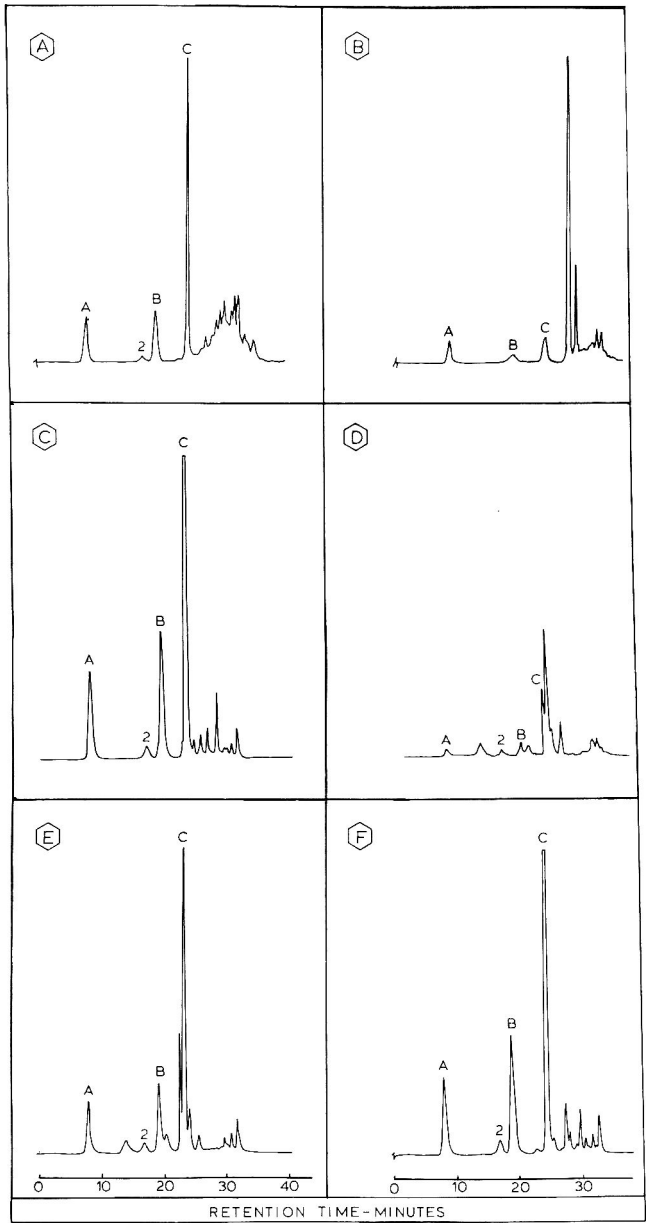

Fig. 2—Anthocyanin profiles of 6 commercial samples of enocyanin. (A) American origin; (B) South African; (C–F) Italian origin. Absorbance 0.64 AUFS. The peak labels are the same as in Fig. 1.

Table 1—Spectral data and HPLC retention times for enocyanin pigments

Peak	A_{440}/A_{max} (%)	Retention time (min)	Pigment
A	32	7.8	delphiniden-3-glucoside
B	30	19.0	petunidin-3-glucoside
C	29	23.1	cyanidin-3-glucoside

containing 0.2% enocyanin. Fig. 1 C and D both show the group of pigments eluting after peonidin-3-arabinoside (Pn-3-Ar) (Peak 5) that are characteristic of enocyanin. These are very obvious in the high sensitivity in 1 C and also in the lower sensitivity setting in 1 D. Replacement of 5% of the cranberry juice with enocyanin is probably the limit of sensitivity for this enocyanin sample, particularly for the A peak, but it can be detected when one also considers the peaks eluting after peak 5.

Fig. 1 B represents a chromatogram from a commercial sample of AppleCranberry juice with enocyanin declared on the label. The apple components do not interfere with the HPLC separation since the 5 cranberry peaks and the 3 enocyanin peaks are obvious. The enocyanin peaks from the enocyanin eluting after peak 5 are also clearly visible. Apple components would not be expected to interfere directly since they do not include compounds with absorbance at 530 nm.

Figure 1 E represents a commercial sample of cranberry juice cocktail which was judged to be adulterated by the method proposed earlier by Francis (1985). The 5 cranberry and the 3 enocyanin peaks as well as the group of peaks eluting after peak 5 are clearly visible. This sample had been opened, reclosed and stored at 45°F for 18 months prior to analysis. Figure 1 F represents a commercial sample of cranberry juice cocktail which had been opened, reclosed, and stored at 45°F for 7 months prior to analysis. The cranberry peaks are clearly visible. Obviously, it is possible with the resin concentration step and the sensitivity of the procedure to obtain results even on old samples. However, old samples are subject to a number of additional variables and would require validation.

SUMMARY & CONCLUSIONS

THE ANTHOCYANIN PIGMENTS in both cranberry juice cocktail and the grape colorant enocyanin can be separated with HPLC using a polymeric reversed phase (Styrene/DVB) column. Two anthocyanins (delphinidin-3-glucoside and petunidin-3-glucoside) were found in all 8 commercial samples of enocyanin but were not present in authentic cranberry juice cocktail samples. A third pigment, similar to cyanidin-3-glucoside but with a longer HPLC retention time, was found in all enocyanin samples but not in cranberry samples. A fourth compound, cyanidin-3-glucoside is found in trace quantities in cranberries and in greater quantities in enocyanin. A group of unidentified pigments in enocyanin elute after the cranberry pigments. When the differences in the anthocyanin chromatograms between cranberry juice and enocyanin are considered, it is possible to detect the replacement of 5% of the expected amount of cranberry juice with an anocyanin solution.

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Production of Riboflavin and Vitamin B_{12} by *Bacillus megaterium* ATCC 13639 and *Enterobacter aerogenes* in Corn Meal

H. J. CHUNG and M. L. FIELDS

ABSTRACT

The % RNV values and available lysine of corn meal were decreased ($P<0.05$) by fermentation with *B. megaterium* ATCC 13639 and *E. aerogenes* but available methionine and tryptophan were significantly increased. Vitamin B_{12} production by *B. megaterium* in corn meal was maximal after 5 days of fermentation at pH 6.0 and 30°C whereas the yield of riboflavin by *E. aerogenes* was the highest after 5 days of fermentation at pH 8.0 at 30°C. There were no differences ($P>0.05$) among the yields of riboflavin by *B. megaterium* in fermented corn meal at different pH and temperatures. Vitamin B_{12} and riboflavin increased most rapidly during the first day of fermentation and continued to increase until the 5th day.

INTRODUCTION

CORN, WHEAT AND RICE provide over one-half of the total calories and total protein for the people in developing countries (Hanson, 1974). However, like other cereals, corn does not supply a complete protein. Lysine, tryptophan and methionine are limiting. Fermentation is the least complex method for improving the nutritional quality of cereal grains and either the natural flora or pure cultures can be used for this process.

Vitamin B_{12} is widely distributed in nature, but higher animals cannot synthesize this vitamin, and it must be obtained directly or indirectly from bacterial sources (Rosenthal, 1968). People in developing countries have a risk of becoming deficient in vitamin B_{12} since they do not have enough animal foods to supply this vitamin; therefore, producing B_{12} in cereal foods is highly desirable.

Another vitamin, riboflavin, is produced in fermentations of corn. Murdock and Fields (1984) reported increases in both vitamin B_{12} and riboflavin in fermented corn meal by a natural lactic acid fermentation. Other desirable nutritional changes that occur in the fermentation of corn meal are improvement in the amino acid balance and bioavailability of minerals. The objectives of this research were to use bacteria that produced vitamin B_{12} and riboflavin to enhance the vitamin content of corn meal. In addition, the influence of these bacteria on the relative nutritive value, lysine, methionine, and tryptophan was determined.

MATERIALS & METHODS

Corn meal preparation

One bag (23 kg) of yellow corn grain *(Zea mays)* was purchased from a local grain distributor and was stored (maximum 3 months) at 4°C until used in this experiment.

Five 2,000-g portions of corn were passed through a 5.66-mm sieve (The W.S. Tyler Co., Cleveland, OH) to remove broken kernels and unwanted debris. Then, the corn was spread on a plastic tray with a fan to blow out other undesirable impurities. The cleaned samples were washed three times in tap water and dried at 50–53°C in a flowing air drier (Model 835, Precision Scientific Co., Chicago, IL) for 24 hr. The dried samples were ground through a 1-mm mesh screen in a Thomas Wiley Laboratory Mill (Model 4, Arthur H. Thomas Co., Philadelphia, PA) and stored in glass jars at 25°C until needed (maximum 3 months). One portion of the whole corn meal was used as a control and stored at 25°C for further analysis.

Authors Chung and Fields are with the Dept. of Food Science & Nutrition, Univ. of Missouri-Columbia, Columbia, MO 65211.

Preparation of fermented corn meal

A slurry of the sample was made by adding 200 mL of tap water to 50g of corn meal. Slurries were steamed in an autoclave at 100°C, after which they were blended with an additional 650 mL of tap water using an electric blender (Model VNS-45820-A, Montgomery Ward & Co., Columbia, MO) to break up clumps. The blended samples were adjusted to optimal pH, determined as described below, using 1N HCl or 1N NaOH and autoclaved for 45 min at 121°C. After cooling, the slurries were inoculated and incubated at the optimal temperature for the production of the vitamins as determined in nutrient broth in preliminary studies.

Bacteria used in fermentations

Bacillus megaterium ATCC 13639 known to produce vitamin B_{12} was obtained from American Type Culture Collection, Rockville, MD. *Enterobacter aerogenes* was isolated in this laboratory by Dyer (1985).

Inoculum for each vitamin production was prepared by subculturing from the stock cultures of *B. megaterium* ATCC 13639 and *E. aerogenes* to 10 ml of nutrient broth. After 24 hr incubation at 30°C, the cells were centrifuged aseptically, the supernatant decanted and the cells suspended in 10 mL of normal saline. Cell counts of *B. megaterium* ATCC 13639 and *E. aerogenes* ranged from $6.3X10^5$ to $1.5X10^6$ and from $1.6X10^8$ to $3.1X10^8$/mL, respectively. Inoculum was used at a level of 1% of the total sample volume.

Fermentation variables

The effect of pH on the production of vitamin B_{12} and riboflavin in corn meal fermented by *B. megaterium* ATCC 13639 and *E. aerogenes* was determined by adjusting samples to pH 5.0, 6.0, 7.0, and 8.0 using 1N HCl or 1N NaOH solutions. The slurries at the four pH levels were incubated for 5 days at 30°C, dried at 50–53°C for 36 hr and re-ground in a Wiley Mill through a 1-mm screen. The ground samples were stored (maximum 3 months) at 25°C in the dark until further analysis.

To determine the optimal temperature for the production of riboflavin and vitamin B_{12}, corn meal samples were inoculated with *B. megaterium* ATCC 13639 and *E. aerogenes* and incubated at 25°, 30°, 35°, and 40°C. Vitamins B_{12} and riboflavin were determined after 5 days of fermentation at the optimal pH for each organism.

To determine the effect of fermentation time on the production of vitamin B_{12} and riboflavin by *B. megaterium* ATCC 13639 and *E. aerogenes*, samples were collected and analyzed each day for 5 days, during fermentation at 30°C at the optimal temperature and at the optimal pH for each organism.

Moisture of the nonfermented and fermented ground corn was determined by the air-oven method (AOAC, 1980).

Nitrogen of the nonfermented and fermented ground corn was determined by the micro-Kjeldahl method (AOAC, 1980).

The pH of the fermented corn meal was measured using a Beckman Zeromatic pH meter. Titratable acidity (TA) was analyzed by titrating 10 mL corn meal slurries with 0.1005N NaOH to an endpoint of pH 8.2.

Table 1—Means[d] and standard deviations for riboflavin and vitamin B_{12} of corn meal fermented with B. megaterium *ATCC 12639 and* E. aerogenes *at different pH values*

	B. megaterium		*E. aerogenes*	
pH	Vitamin B_{12} (μg/100 g)	Riboflavin (mg/100 g)	Vitamin B_{12} (μg/100 g)	Riboflavin (mg/100 g)
5.0	3.70 ± 1.03^{b}	0.16 ± 0.01^{a}	3.16 ± 0.47^{a}	0.32 ± 0.02^{c}
6.0	4.93 ± 0.34^{a}	0.19 ± 0.03^{a}	3.74 ± 0.22^{a}	0.36 ± 0.01^{b}
7.0	3.20 ± 0.26^{bc}	0.18 ± 0.01^{a}	4.17 ± 1.32^{a}	0.38 ± 0.02^{ab}
8.0	2.11 ± 0.50^{c}	0.15 ± 0.02^{a}	4.56 ± 0.77^{a}	0.41 ± 0.04^{a}

[a-c] N = 4. Where letters differ (a-c) within a column, means differ significantly (P<0.05) from each other.

[d] Calculated on dry weight basis. Fermented at 30°C for 5 days.

Table 2—Effect of temperature on the production of vitamin B_{12} and riboflavin in corn meal fermented with B. megaterium *ATCC 13639 and* E. aerogenes[c]

	B. megaterium		*E. aerogenes*	
Temp. (°C)	Vitamin B_{12} (μg/100 g)	Riboflavin (mg/100 g)	Vitamin B_{12} (μg/100 g)	Riboflavin (mg/100 g)
25	3.81 ± 1.58^{a}	0.21 ± 0.07^{a}	4.17 ± 0.54^{a}	0.33 ± 0.03^{a}
30	5.08 ± 0.45^{a}	0.22 ± 0.07^{a}	4.74 ± 0.45^{a}	0.34 ± 0.02^{a}
35	3.84 ± 0.60^{a}	0.20 ± 0.02^{a}	4.03 ± 0.40^{b}	0.32 ± 0.03^{a}
40	2.96 ± 0.57^{a}	0.18 ± 0.02^{a}	3.80 ± 0.56^{b}	0.31 ± 0.02^{a}

[a,b] N = 4. Where letters (a-b) differ within a column, means differ significantly (P<0.05) from each other.

[c] Calculated on dry weight basis. Fermented at pH 6.0 for 5 days.

Determination of percent relative nutritive value (% RNV)

The percent relative nutritive values of the nonfermented and fermented corn meal were determined according to the method of Stott et al. (1963).

Determination of available lysine, methionine and tryptophan

Test materials were prepared according to Ford (1964). The prepared samples were analyzed for availability of the amino acids, lysine, methionine, and tryptophan, by microbiological assays (Difco, 1977).

Determination of phytase activity

To determine the extent of the hydrolysis of phytic acid by bacterial phytase from *B. megaterium* ATCC 13639 and *E. aerogenes*, 1L of nutrient broth was mixed with 1.8g of phytic acid (sodium salt, Sigma Chemical Co., St. Louis, MO). Ten milliters of the mixture was added to 12 test tubes (20 X 150 mm). The samples were autoclaved at 121°C for 15 min and incubated for 5 days at 30°C.

Inorganic phosphorus was determined by the Fiske and Subbarow (1925) method. Phytic acid was calculated on the basis of a 28.2% phosphorus content in phytic acid.

Vitamin determination

The assay of vitamin B_{12} was according to the Association of Vitamin Chemists (1966) except the extraction was done according to Bell (1974). Difco media for both B_{12} and riboflavin analyses were used (Difco, 1977). Riboflavin was determined according to the procedure of the Association of Vitamin Chemists (1966).

Statistical analysis

Analysis of variance was determined according to Snedecor and Cochran (1980). These analyses were accomplished by means of a computer program developed by Payton (1985). When significant (P<0.05) differences were found, Duncan's (1955) new multiple range test was used to locate the means that differed. Standard deviations were also determined and are reported with the means.

RESULTS & DISCUSSION

Effect of pH

The effects of pH on the production of vitamin B_{12} and riboflavin by *B. megaterium* ATCC 13639 and *E. aerogenes* in fermented corn meal are listed in Table 1. Vitamin B_{12} and riboflavin production by *B. megaterium* ATCC 13639 was highest at initial pH 6.0; however, riboflavin productions at different pH values were not different (P>0.05). There were no differences (P>0.05) in vitamin B_{12} produced by *E. aerogenes* at different pH values. The greatest production of riboflavin by *E. aerogenes* was obtained at pH 8.0.

Effect of temperature

Table 2 presents data indicating the effect of fermentation temperature upon the production of vitamin B_{12} and riboflavin in corn meal fermented with *B. megaterium* ATCC 13639 and *E. aerogenes*. The highest amounts of vitamin B_{12} and riboflavin were obtained when the samples were fermented with *B. megaterium* ATCC 13639 at 30°C, but there were no differences (P>0.05) among vitamin B_{12} and riboflavin yields at different temperatures.

The data also indicated that the production of vitamin B_{12} and riboflavin by *E. aerogenes* was the highest at 30°C. The quantity of vitamin B_{12} synthesized by *E. aerogenes* at 25°C and 30°C was different (P<0.05) from that at 35°C and 40°C, whereas riboflavin production did not differ significantly at any of the temperatures tested.

Table 3—Means[e] and standard deviations for vitamin content of corn meal fermented with B. megaterium *ATCC 13639 and* E. aerogenes *at the optimal conditions*

Corn meal	Vitamin B_{12} (μg/100 g)	Riboflavin (mg/100 g)
Nonfermented control	0.07 ± 0.02^{d}	0.11 ± 0.01^{d}
Fermented with *B. megaterium* ATCC 13639[f]	5.04 ± 0.44^{b}	0.18 ± 0.01^{c}
Fermented with *E. aerogenes*[g]	4.35 ± 0.19^{c}	0.38 ± 0.01^{b}
Fermented with mixed culture[h]	5.57 ± 0.12^{a}	0.45 ± 0.05^{a}

[a-d] N = 4. Where letters (a-d) differ within a column, means differ significantly (P<0.05) from each other.

[e] Calculated on dry weight basis.

[f] Fermented at pH 6.0, 30°C for 5 days.

[g] Fermented at pH 8.0, 30°C for 5 days.

[h] Fermented with mixed culture (1:1, v/v) of *B. megaterium* ATCC 13639 and *E. aerogenes* at pH 7.0, 30°C for 5 days.

Effect of time

Vitamin B_{12} increased most rapidly during the first day of fermentation (0.10 μg/100g to 2.3 μg/100g) for *B. megaterium* and from 0.15 μg/100g to 3.0 μg/100g for *E. aerogenes*. Riboflavin, like vitamin B_{12}, also increased most rapidly during the first day of fermentation (0.11 mg/100g to 0.18 mg/100g for *B. megaterium and* 0.11 mg/100g to 0.33 mg/100g for *E. aerogenes*). There were no differences (P<0.05) among samples fermented for 5 days.

In natural fermentation of corn meal, however, Murdock and Fields (1984) found that during lactic acid fermentation of corn meal, vitamin B_{12} and riboflavin declined between day two and three and increased between day three and four. This indicated the presence of microorganisms requiring vitamin B_{12} for growth. In the experiments reported here, only *B. megaterium* ATCC 13639 was present so there was no decline after production of vitamin B_{12} or riboflavin.

Relative yields

Yields do not account for total solids loss due to microbial activity; therefore, the values are not absolute but are relative yields of vitamins.

The quantities of vitamin B_{12} and riboflavin produced by *B. megaterium* ATCC 13639 (at pH 6.0, 30°C for 5 days) and *E. aerogenes* at pH 8.0, 30°C for 5 days) at the optimal conditions (determined previously in nutrient broth) were 5.04 μg/100g and 0.18 mg/100g, and 4.35 μg/100g and 0.38 mg/100g, respectively (Table 3). These vitamin productions by both organisms were twice higher than in natural lactic acid fermentation

Table 4—Means[e] and standard deviations for percent relative nutritive value (% RNV) of nonfermented and fermented corn meal

Corn meal	RNV (%)
Nonfermented control	88.7 ± 0.54[e]
Fermented with *B. megaterium* ATCC 13639[f]	79.8 ± 0.00[c]
Fermented with *E. aerogenes*[g]	77.6 ± 0.95[d]
Fermented with mixed culture[h]	82.9 ± 1.16[b]

[a-d] N = 4. Where letters (a-d) differ, means differ significantly (P<0.05) from each other.
[e] Calculated on dry weight basis.
[f] Fermented at pH 6.0, 30°C for 5 days.
[g] Fermented at pH 8.0, 30°C for 5 days.
[h] Fermented with mixed culture (1:1, v/v) of *B. megaterium* ATCC 13639 and *E. aerogenes* at pH 7.0, 30°C for 5 days.

by other investigators (Murdock and Fields, 1984; Tongnual, 1982).

Vitamin B_{12} and riboflavin produced by the mixed culture of these two organisms were higher (P<0.05) than in pure cultures. All samples fermented with different organisms were significantly higher in vitamin B_{12} and riboflavin than the nonfermented control (Table 3). According to Rosenthal (1968), vitamin B_{12} is not found to any extent in plant materials including cereal grains. Therefore, the vitamin B_{12} activity measured in nonfermented control would be due to the assay method used and this may not be a true indication of the vitamin B_{12} activity. Since Garibaldi et al. (1953) reported that the yields of vitamin B_{12} were influenced by the history of the inoculum, fresh inoculum was applied to the samples in this study. Although they also found that increased aeration of fermentation broth further increased yields of vitamin B_{12}, the samples in this study were not shaken.

Relative nutritive value

Data on the % RNV of nonfermented and fermented corn meal are presented in Table 4. The % RNV value in fermented corn meal was lower (P<0.05) than that in non-fermented corn meal. This indicated that the limiting essential amino acids did not increase during fermentation of whole ground corn meal.

The % RNV is an indication of amino acid balance (Evancho et al., 1977). According to Rosen and Fernell (1956), the efficiency of protein utilization can be expressed by the ratio of the number of organisms per mL of culture medium to the amount of ammonia nitrogen present after four days incubation. The amino acid requirement for *T. furgasoni* is comparable to rats and human beings (Evancho et al., 1977). Therefore, measurement of *T. furgasoni* growth was used to evaluate the % RNV of bacterial fermented corn meal. Bacteria used singly or in combination of two did not improve the % RNV. However, the species *(Lactobacillus fermentum, Bacillus cereus* and *Pseudomonas maltophilia)* and numbers of bacteria made a difference (Nanson and Fields, 1982). These data supported their findings.

The decrease in % RNV did not follow the increases in available amino acids and vitamins found in other studies. Perez (1980) showed that the % RNV of the corn meal fermented by *Candida tropicalis* ATCC 13639 did not increase (P>0.05). Differences in % RNV in this study compared with other natural lactic fermentation studies can be attributed to different microorganisms involved in the fermentation. Since the nonfermented control had a higher value for the % RNV, the data also suggested that the nutrient value of the corn used in this experiment was higher than in other studies. In natural lactic acid fermentation, however, Hamad (1978), Tongnual et al. (1981) and Zamora (1978) reported significant % RNV increase when the cereals and legumes were used.

It appears that improving the % RNV is more difficult. One or two species of bacteria in the fermentation are not enough to bring the change in the amino acid balance.

Available amino acids

Available methionine and tryptophan of corn meal were increased (P<0.05) by fermentation with pure or mixed cultures

Table 5—Means[e] and standard deviations for available lysine, methionine and tryptophan of nonfermented and fermented corn meal

	Amino acid		
Corn meal	Lysine (mg/100 g)	Methionine (mg/100 g)	Tryptophan (mg/100 g)
Non-fermented control	39.00 ± 0.84[a]	11.53 ± 0.32[d]	3.76 ± 0.08[d]
Fermented with *B. megaterium* ATCC 13639[f]	34.76 ± 0.70[b]	23.79 ± 0.29[b]	6.33 ± 0.06[b]
Fermented with *E. aerogenes*[g]	26.79 ± 0.79[c]	21.47 ± 0.19[c]	5.18 ± 0.02[c]
Fermented with mixed culture[h]	40.24 ± 1.13[a]	28.76 ± 0.25[a]	7.43 ± 0.09[a]

[a-d] N = 4. Where letters (a-d) differ within a column, means differ significantly (P<0.05) from each other.
[e] Calculated on dry weight basis.
[f] Fermented at pH 6.0, 30°C for 5 days.
[g] Fermented at pH 8.0, 30°C for 5 days.
[h] Fermented with mixed culture (1:1, v/v) of *B. megaterium* ATCC 13639 and *E. aerogenes* at pH 7.0, 30°C for 5 days.

of *B. megaterium* ATCC 13639 and *E. aerogenes* (Table 5). However, available lysine decreased (P<0.05) in corn meal fermented with *B. megaterium* ATCC 13639 and *E. aerogenes*. Decreases in available lysine of fermented samples may be related to the need by both bacteria to produce this amino acid. However, the bacteria together did not decrease lysine. However, with mixed cultures less was utilized in growth and hence the value was near the control indicating some possible lysine synthesis. The fact that there was less lysine in samples fermented by each bacterium and more in the mixed culture corroborated the % RNV data.

According to Benton et al. (1956), an excess of the second limiting amino acid in the diet increases the requirement of the rat for the first limiting amino acid. Also, Brickson et al. (1948) and Dien et al. (1954), using *Lactobacillus arabinosus* 17-5 and *Leuconostoc dextranicum* 8086, demonstrated that high levels of leucine and valine inhibited the utilization of isoleucine for growth and that excesses of leucine and isoleucine inhibited the utilization of valine in a similar manner. With these facts in mind, it may be hypothesized that in this investigation, the increased level of tryptophan intensified the limiting effect of lysine. The first and second limiting amino acids were lysine and tryptophan, respectively.

Available methionine and tryptophan of fermented corn meal with the mixted culture were higher (P<0.05) than those in non-fermented control and in fermented corn meals with pure cultures of these two organisms. Both bacteria may have the pathways to produce these amino acids and more was produced than needed for growth. In the case of methionine and tryptophan, mixed cultures produced increases (P<0.05) in the amounts of each amino acid.

Tongnual and Fields (1984) reported that the availability of lysine, methionine and tryptophan of corn and corn-soybean mixtures was increased (P<0.05) by natural lactic acid fermentation. The magnitude of the increases was two- to threefold for lysine, methionine, and tryptophan. These differences may be indicative of the different microorganisms involved in this investigation contrasted with their study. Hamad and Fields (1979), Kazanas and Fields (1981), and Tongnual et al. (1981) obtained similar results for other cereal grains. In their studies on natural lactic fermentation, these investigators used naturally occurring microflora in corn meal. Tongnual et al. (1981) showed that proteolytic bacteria were responsible for hydrolyzing the proteins. This allowed an increase in available amino acids during the corn meal fermentation.

Titratable acidity (TA) and pH

The titratable acidity of fermented corn meal with *B. megaterium* ATCC 13639 increased progressively to 0.039% at the

Table 6—Means and standard deviations for phytase activity of B. megaterium *ATCC 13639 and* E. aerogenes[d]

Microorganism	Phytate-phosphorus (mg/tube)	Phytic acid[e] (mg/tube)	Digestion[f] (%)
Control (uninoculated)	0.51 ± 0.01[a]	1.80 ± 0.02	- - - -
B. megterium ATCC 13639	0.26 ± 0.00[c]	0.91 ± 0.01	49.52
E. aerogenes	0.38 ± 0.01[b]	1.36 ± 0.01	25.49

[a-c] N = 4. Where letters (a-c) differ within a column, means differ significantly ($P<0.05$) from each other.

[d] Tubes were incubated at 30°C for 5 days.

[e] Phytic acid was determined by a conversion factor of 3.55 (28.2% phosphorus in phytic acid).

[f] Percent digestion was calculated using the formula:

$$\% \text{ digestion} = [(0.51 - \text{remaining phytate-phosphorus})/0.51)] \times 100.$$

5th day of fermentation, whereas the pH decreased from pH 6.45 to pH 5.68.

Earlier studies (Fields et al., 1981; Murdock and Fields, 1984; Zamora and Fields, 1979) showed titratable acidity increased to the 4th day and then declined between day 4 and 5 in a natural lactic acid fermentation. The decline in pH in their fermentations were thought to be due to film yeasts and molds that grew in or on fermented samples and subsequently used lactic acid for growth.

Titratable acidity of fermented corn meal with *E. aerogenes* slowed abruptly at 0.074% during the first day of fermentation corresponding to a drop in pH from pH 7.72 to pH 5.59. After the 5th day of fermentation, the titratable acidity and pH were 0.099% and 5.30, respectively. Because of the less acidic fermentation, the possible use of the fermented material in foods would probably be accepted more readily than the natural lactic acid corn meal.

Activity of bacterial phytase

The microbial phytase activities of *B. megaterium* ATCC 13639 and *E. aerogenes* are given in Table 6. The phytase activity of *B. megaterium* ATCC 13639 was higher ($P<0.05$) than that of *E. aerogenes*. This indicates that the fermented corn meal with *B. megaterium* ATCC 13639 might have more available minerals than that with *E. aerogenes* and the non-fermented control, since the active phytase reduced the phytic acid of corn meal and probably increased the bioavailability of minerals.

In a similar study, Lopez (1982) showed that twenty proteolytic bacteria isolated from fermented corn meal by Tongnual et al. (1981) had phytase activity. Tongnual (1982) reported that phytic acid in corn meal was decreased ($P<0.05$) by fermentation, thus making more iron and phosphorus available. Lopez (1982) also explained that phytase produced by bacteria during fermentation was probably responsible for the reduction of phytic acid in corn meal.

B. megaterium ATCC 13639 and *E. aerogenes* have potential for the production of vitamin B_{12} and riboflavin in the fermentation of corn meal. Since both bacteria had phytases, mineral availability should be increased. Since these bacteria decreased lysine, an organism producing an excess of lysine should be included in future research.

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Functional Properties of Drum-Dried Chickpea (*Cicer arietinum* L.) Flours

MARINA CARCEA BENCINI

ABSTRACT

The chemical and functional properties of three precooked flours prepared from citric acid- and sodium bicarbonate-treated chickpeas were compared with those of raw chickpea and soybean flours. The nitrogen solubility and foaming capacity of the precooked flours were markedly reduced by processing. The acid-treated flours showed higher values of oil absorption and viscosity than the sodium bicarbonate-treated flour, but the latter had a higher bulk density. Even at 10% concentration, the acid-treated flour slurries manifested very low viscosities (about 11 centipoises). No significant difference was detected in the water absorption and gelation capacity of the precooked flours.

INTRODUCTION

PROCESSING PULSES into precooked flours to incorporate into traditional foods (bakery products, pasta) or new products (breakfast cereals, snacks, dips, baby foods, soups and beverages) could extend the utilization of grain legumes and increase their consumption in the industrialized countries (White and Kon, 1972; Kon and Burtea, 1979; Anonymous, 1980).

Several authors have reported the processing and physicochemical properties of precooked flours from various pulses. Instant flours were prepared by soaking the seeds, cooking, slurrying them and then drum drying the slurry (Boggs et al., 1964; Bakker-Arkema et al., 1967; Burr et al. 1969; Guadagni et al., 1975; Onayemi and Potter, 1976). The flour obtained by this method retained the characteristic flavor of the legume from which it was prepared. Kon et al. (1970) produced precooked flours with an exceptionally mild beany flavor by blending legume flours with acidified water, cooking and neutralizing the acid slurry and then by drum drying it. Fung Miller et al. (1973) and Kon et al. (1974) applied the same process to various legumes. Okaka and Potter (1979) published a variation in which legume seeds were soaked in acidified water, steam blanched, slurried and drum-dried.

Chickpea (*Cicer arietinum* L.) ranks fifth in order of world production, after soybean, groundnut, haricot bean and pea, and is consumed worldwide.

There is little available information on precooked chickpea flours. This research was undertaken to study the effect of different processing conditions on the functional properties of precooked chickpea flours obtained by the three reported methods. The knowledge of these properties was important to predict the possible use of the flours in traditional and new processed foods.

MATERIALS & METHODS

CHICKPEA SEEDS (*Cicer arietinum* L.) (10 kg) were purchased in Italy but they originated from Turkey (1983 harvest). The average weight of each grain was 0.5g ± 0.07g. A sample of 100g was ground in a coffee mill to a 60 mesh raw flour which was used for the determination of proximate composition and functional properties. The remainder of the seeds was used for the preparation of the precooked flours.

Author Carcea Bencini was with the Dept. of Food Science, Queen Elizabeth College, Univ. of London, Campden Hill Road, London W8, England. Present address is Via Nemorense, 177, 00199 Roma, Italy.

Precooked flour from $NaHCO_3$-treated seeds (T-1)

Chickpea seeds were soaked overnight in a 0.5% solution of $NaHCO_3$ (1:5 w/v, 24°C). Preliminary experiments had shown, in accordance with the literature (Varriano-Marston and De Omana, 1979; Caro Bueno et al., 1980; Edijala, 1980; Kadam et al., 1981) that 0.5% of $NaHCO_3$ in the soaking water considerably reduced cooking time. The seeds were drained and cooked in distilled water (1:4 dry seeds w/v) at 100°C for ca 30 min. The cooked seeds and the broth were slurried in a food processor (Kenwood, Peerless & Ericsson Ltd., England).

Precooked flour with citric acid treatment (T-2)

Chickpea seeds were ground in a coffee mill to a 40 mesh flour. A 0.01% solution of citric acid was added to the flour (1:3 w/v) to produce an acid slurry (pH 3.4) which was cooked for 5 min then adjusted to pH 7 with $NaHCO_3$ and cooked for further 45 min. The pH of the slurry at which no beany flavor or odor could be detected (pH 3.4) had been selected in a preliminary experiment during which raw chickpea flour was blended with acidified water to produce slurries of pH 6.5, 4.1, 3.4 and 3.0. The slurries were cooked for 5 min, adjusted to pH 7 with $NaHCO_3$ and cooked further for 45 min. Five trained panelists were presented with coded samples of the cooked slurries (cooled to room temperature, in disposable plastic cups) and asked to evaluate them for their beaniness by the scoring method. The test was conducted in individual booths. In the pH 3.4 and 3.0 slurries the panelists could not detect any beany flavor or odor.

Precooked flour from citric acid-treated seeds

Chickpea seeds were soaked overnight in a 0.01% solution of citric acid (pH 3.4) (1:5 w/v, 24°C). The soaked seeds were then steam cooked for about 20 min. To determine the degree of cooking, five seeds were withdrawn every 5 min and checked by the 'visual examination' method of Williams et al. (1983). The steamed chickpeas were mashed in the food processor. Some distilled water had to be added to the puree in order to liquify it for easier drum drying (water content of the puree ca 77%). The slurry was then homogenized for 1 min in a Waring Blendor at high speed.

Drum drying

The slurries were dried on an atmospheric, single drum drier (Richard & Sons Ltd., England) equipped with a 20 × 20 cm drum, variable speed drive and take off rolls. The drum was heated at a steam pressure of 1.4 kg/cm^2 and the drying time was 20 sec. The dried flakes were ground in a coffee mill to 60 mesh flours which were air-packed, hermetically sealed in polyethylene bags and stored at 4°C.

Composition

Moisture, ash, ether soluble material, crude fiber and crude protein (N × 6.25) were determined according to standard AOAC (1984) methods. Total carbohydrate was determined by difference.

Functional properties

For some tests a soybean flour (60 mesh, moisture 8.68%), obtained by grinding soybean seeds marketed in U.K. in a coffee mill, was used for comparison. Unless otherwise stated, all the experiments were conducted at room temperature (24°C) and all the weights reported are dry weights.

Bulk density was determined according to Wang and Kinsella (1976) using samples of 20g (actual weight) and 50 mL graduated cylinders. Bulk density was calculated as g(actual weight)/mL.

Table 1—Proximate composition of raw and precooked chickpea flours[a]

Component	Raw flour (%)	Precooked flours (%) T-1	T-2	T-3
Moisture	7.40	2.61	6.38	6.62
Total solids	92.60	97.39	93.62	93.38
Crude protein (N × 6.25)	21.37	20.72	21.75	21.50
Ether extract	7.17	8.09	6.85	7.23
Ash	2.98	2.04	3.26	2.59
Crude fiber	2.16	2.19	2.08	2.23
Carbohydrate (by diff.)	58.92	64.35	59.68	59.83

[a] All the values are the means of duplicate determinations on dry weight basis.

Table 2—Bulk density, water and oil absorption of raw soybean flour, raw chickpea flour and precooked chickpea flours[a]

Sample	Bulk density (g/mL)	Liquid absorption (mL/g dry matter) Water	Oil
Soybean flour	0.43[d]	2.77[c]	1.93[b]
Chickpea flour	0.47[c]	1.72[d]	1.40[d]
Flour T-1	0.61[b]	3.67[b]	1.32[d]
Flour T-2	0.50[c]	3.75[b]	1.61[c]
Flour T-3	0.49[c]	3.67[b]	1.57[c]

[a] All the values are the means of triplicate determinations.
[b,c,d] In each column, any two means not followed by the same superscript are significantly different ($p \leq 0.05$).

Water and oil absorption capacities were determined according to Beuchat (1977). Samples of 2g were mixed with 20 mL distilled water or corn oil (Mazola, CPC, England) in 50 mL centrifuge tubes. Each slurry was vortexed for 1 min, allowed to stand for 30 min and then centrifuged at 2208 × *g* for 30 min. The results were expressed as mL of liquid retained per g of sample.

Nitrogen solubility was determined in duplicate at various pH values (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0) according to Betschart (1974). However, in this study, 13.5 mL of distilled water were added to 100 mg samples, the final volume was brought to 15 mL with distilled water, and the samples were centrifuged at 334 × *g* for 15 min. Nitrogen Solubility Index (NSI) was calculated as percent nitrogen present in the supernatant.

Foaming capacity and foam stability were also determined on defatted chickpea and soybean flours. Defatting was accomplished by extraction with hexane (1:10 w/v) for 6 hr at room temperature. The defatted flours were air-dried (26°C) to constant weight.

Foaming capacity was measured according to Coffmann and Garcia (1977). Sample dispersions of 3% (w/v) in distilled water were adjusted to pH 7.0 with 0.1N NaOH, whipped for 5 min with a food mixer (Major Mixer S, 3-speed, Moulinex, France) at the highest speed and then poured into 250 mL graduated cylinders. The results were expressed as per cent increase in volume. Foam volumes were recorded at 5, 15, 30, 45, 60, 90, 120, and 180-min intervals to study the foam stability of the samples.

Gelation properties were investigated according to Coffmann and Garcia (1977). Sample dispersions of 2, 4, 6, 8, 10, 12, 14, 16, and 18% (w/v) were prepared in 300 mL distilled water. Each dispersion was adjusted to pH 7.0 with 0.1N NaOH and mixed in the Waring Blendor at the highest speed for 2 min. The dispersions were poured into test tubes in 5 mL aliquots (three test tubes for each concentration), heated to 100°C in a water bath for 1 hr and cooled to 4°C on an ice bath. The lowest concentration at which all the triplicates formed a gel which did not collapse or slip from the inverted test tube was reported as the Least Gelation Concentration (LGC).

Viscosity was studied on sample dispersions of 1, 2, 5, and 10% (w/v) in distilled water. Each dispersion was adjusted to pH 7.0 with 0.1N NaOH, magnetically stirred for 1 hr and its viscosity was then determined with a Haake 'Rotovisco' RV3 coaxial viscometer (Gebrüder Haake, Karlsruhe, Germany) measuring the shear stress developed at shear rates ranging from 2 to 1694/sec. All the measurements were done in duplicate.

RESULTS & DISCUSSION

Color, flavor and water dispersibility

Flour T-1 was light yellow while flours T-2 and T-3 had a distinctly different buff color. All the flours were easily dispersed in distilled water at 24°C and the pH of the slurry was 7.1 for flours T-1 and T-2, and 6.4 for flour T-3.

Flavor evaluation of the precooked flours (50g of flour in 200 mL boiling water with 0.5% NaCl w/v) as slurries by the panel, indicated that flours T-2 and T-3 had lost most of their characteristic chickpea flavor and odor in comparison with flour T-1.

Composition

An analysis of variance on the values of the proximate composition of raw chickpea flour and precooked flours (Table 1) indicated that there was no significant difference ($p \leq 0.01$) in the crude protein and crude fiber of the samples. There was a significant difference between the moisture of flour T-1 and that of flours T-2 and T-3. The different moisture of the slurries applied to the drum drier was partly responsible for the different moisture of the precooked flours. However, in the case of flour T-2 the larger amount of free pregelatinized starch in the cooked slurry, as found by Kon et al. (1971) for California small white beans and soybeans, could account for less efficient drum drying and therefore, for increased moisture in the precooked flours. Flours T-1 and T-3 lost part of their ash during the soaking of the seeds. The $NaHCO_3$ solution leached more ash than the citric acid solution. Flour T-2 gained ash because of the addition of $NaHCO_3$ to the raw flour.

Bulk density, water and oil absorption

An ANOVA and Tukey's Test (Snedecor, 1956) were performed on the values of bulk density, water and oil absorption of soybean flour, raw chickpea flour and precooked flours (Table 2). There was no significant difference between the bulk density of the raw chickpea flour and those of flours T-2 and T-3. Flour T-1 showed the highest bulk density. Kon et al. (1974) also reported that acidified precooked California small white and pinto bean flours were less dense then the 'regular' (untreated) flours probably because of the larger amount of free, pregelatinized starch in the acidified cooked slurries. Soybean flour was significantly less dense than raw chickpea flour.

All the precooked flours had more than double water retention values than the raw flour (Table 2). However, there was no significant difference ($p \leq 0.01$) in the water absorption capacities of the precooked flours. These results agree with those of Narayana and Narasinga Rao (1982) who reported that heat processed winged bean flour had higher water absorption capacity than the raw flour. Protein denaturation, starch gelatinization and swelling of the crude fiber which may occur during heat treatment, could all be responsible for the increased water absorption capacity of the precooked flours. Soybean flour showed a higher water absorption capacity than raw chickpea flour and the reported value (2.77 mL/g) is identical to the value obtained by Fleming et al. (1974) with the same technique.

Raw chickpea flour showed a lower oil binding capacity than soybean flour (Table 2). Kinsella (1976) explained the mechanism of fat absorption as a physical entrapment of oil and several authors have related the oil absorption capacity to the nonpolar side chains of the proteins. In particular, the different protein concentration, the content of nonpolar amino acids, their sequence in the polypeptide chains and the different conformational features of the proteins could all be an explanation for different fat retaining characteristics. Processing increased the oil absorption capacity of flours T-2 and T-3 while apparently not having any effect on flour T-1. There was no significant difference between the oil absorption capacity of flours T-2 and T-3.

Nitrogen solubility

Chickpea proteins had a point of minimum dispersion at pH 4.0 (ca 10% nitrogen solubility) and their solubility increased

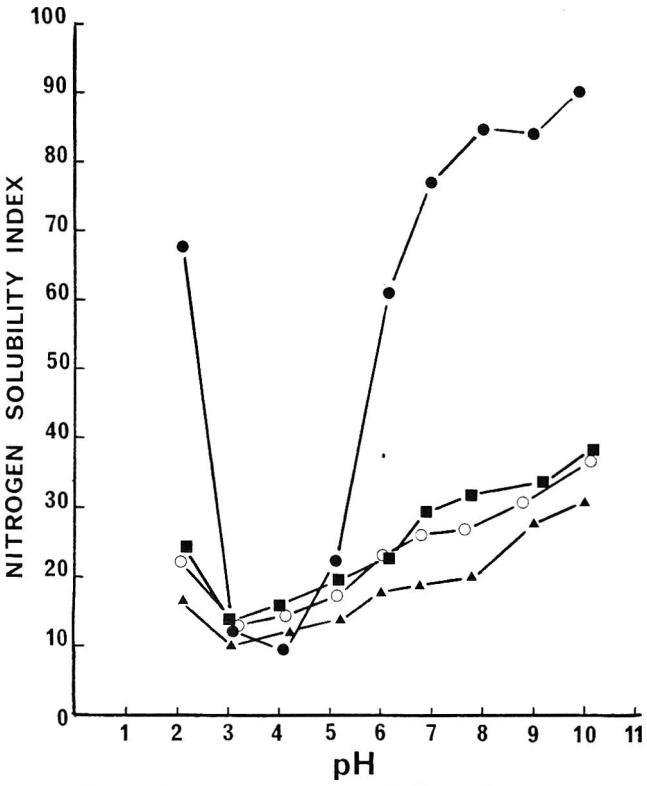

Fig. 1—Nitrogen solubility profiles of raw and precooked chickpea flours : —●— raw flour; —▲— flour T-1; —■— flour T-2; —○— flour T-3.

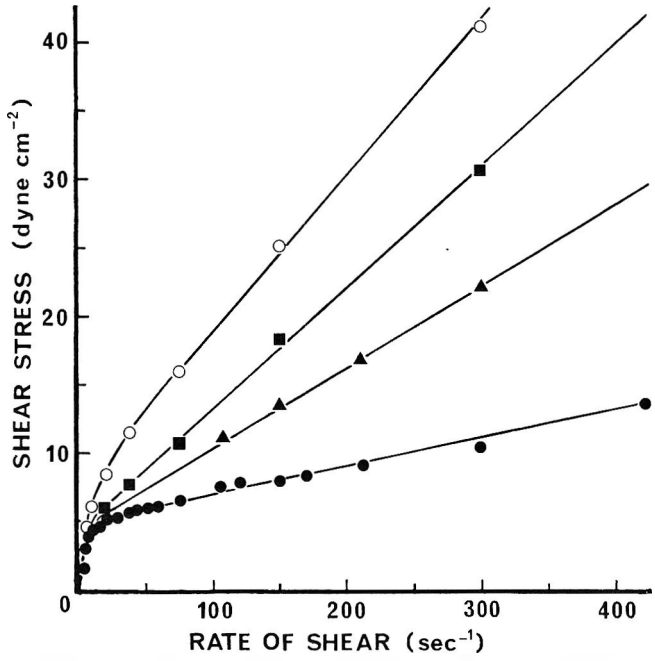

Fig. 2—Flow curves of 10% slurries (distilled water, pH 7.0) of raw and precooked chickpea flours at 24°C : —●— raw flour; —▲— flour T-1; —■— flour T-2; —○— flour T-3.

at lower and higher pH values (Fig. 1). A 91% solubility was obtained at pH 9.8. The NSI curve of the chickpea proteins was similar to the profiles reported by other authors for different pulses (Hang et al., 1970; Beuchat, 1977; Narayana and Narasinga Rao, 1982). The NSI of the precooked flours was much lower than the NSI of the raw flour at pH values above 5.0 and below 3.0 as a consequence of heat treatment. Heat has been reported as one of the factors which negatively affects nitrogen solubility in processed products (McWatters and Holmes, 1979; Narayana and Narasinga Rao, 1982). All the precooked flours showed a minimum NSI at about pH 3.0 (ca 13% nitrogen solubility) and their NSI increased with increasing pH up to a maximum of about 40% for flour T-2. At every pH studied, flour T-1 showed lower values of NSI than flours T-2 and T-3 which gave very similar results. Differences in the NSI profile of the precooked flours could result mainly from the effects of different processing conditions (soaking medium, length of heating treatment) upon the physical state of the proteins.

Foaming properties

Under the conditions of this study (3% aqueous dispersion, pH 7.0, 24°C) chickpea flour showed a higher whippability than soybean flour with the volume increase for chickpea flour being more than three times that for soybean flour (Table 3). The effect of lipids on foaming capacity and foam stability of raw chickpea and soybean flours was also studied. Removal of lipids with hexane improved the foaming capacity of both the chickpea and soybean flours (Table 3). The foaming capacity of defatted soybean flour in this investigation (355%) was in agreement with the value reported by Sosulski et al. (1976) for dehulled defatted soybean flour (375%). Chickpea flour showed less foam stability than soybean flour (Table 3). Defatting markedly increased foam stability of chickpea flour. Processing of chickpeas had a negative effect on the whippability of the precooked flours (Table 3). Lawhon et al. (1972) and Narayana and Narasinga Rao (1982) have reported the negative effect of heat treatment on the foaming characteristics of glandless cottonseed flour and winged bean flour, respectively. Several authors have suggested a direct relationship between foaming capacity and protein solubility of leguminous and non-leguminous products (Lawhon et al., 1972; Yasumatsu et al., 1972; Wu and Inglett, 1974; Nath and Narasinga Rao, 1981; Anusuya Devi and Venkataraman, 1984). A comparison between NSI and foaming capacity of raw chickpea flour and precooked flours (Fig. 1 and Table 3) indicates that at pH 7.0 chickpea flour has a higher NSI and foaming capacity than the precooked flours. Flours T-2 and T-3 gave close results for NSI and foaming capacity while flour T-1 gave lower values in both tests.

Gelation

All the precooked flours formed a strong gel at a minimum concentration of 14% while raw chickpea flour required a minimum concentration of 16%. The latter value was higher than the values obtained for bean flours (10–14%) (Deshpande et al., 1982), for lupin flour (14%) (Sathe et al., 1982a) and for black gram flour (12%) (Sathe et al., 1983) but lower than that for winged bean flour (18%) (Sathe et al., 1982b). Differences in the gelling ability of different species of pulses could be due not only to differences in protein, but also to the nature of proteins. Higher proportion of globular proteins could contribute to higher LGC values (Sathe et al., 1982b). According to Narayana Rao and Rajagopal Rao (1974), globulins account for 60–80% of the total protein in chickpea seeds. This could be one of the reasons why chickpeas have higher LGC than other pulses. Protein denaturation and starch gelatinization occurring during heat treatment of the precooked flours could facilitate the formation of a stronger gel matrix at lower concentrations.

Viscosity

The study of the flow characteristics of 10% slurries of raw chickpea flour and precooked flours indicated that all the slurries had a very weak pseudoplastic behavior (Fig. 2). At rates of shear over 75/sec, all the samples showed a linear relationship between shear stress and rate of shear (i.e., Newtonian

Table 3—Foaming capacity and foam stability of raw soybean flours, raw chickpea flours and precooked chickpea flours[a]

Sample	Volume increase (%)	Volume of foam (mL) after time (min)								
		1	5	15	30	45	60	90	120	180
Soybean flour	58	69	62	60	58	55	44	34	25	19
Soybean defatted flour	355	455	447	420	396	386	382	373	368	359
Chickpea flour	193	222	215	191	118	42	42	32	32	32
Chickpea defatted flour	285	375	340	315	303	294	289	272	270	260
Flour T-1	5	10	9	8	6	4	3	3	3	2
Flour T-2	7	11	9	8	7	6	6	6	5	4
Flour T-3	8	11	9	8	7	7	6	5	4	3

[a] All the values are the means of duplicate determinations.

Table 4—Apparent viscosity of raw and precooked chickpea flour slurries of different concentration (w/v)

Sample	Apparent viscosity (cp)[a]			
	1%	2%	5%	10%
Chickpea flour	3.69	3.82	3.88	4.45
Flour T-1	3.75	3.94	4.20	7.60
Flour T-2	3.94	4.20	5.22	10.18
Flour T-3	3.94	4.20	5.41	11.96

[a] Apparent viscosity at a 1198/sec shear rate, room temperature (24°C). All the values are the means of duplicate determinations.

behavior). Below 10/sec rate of shear, all the slurries showed the same shear stress values. Studies of 12% slurries confirmed previous results. The precooked flour slurries gave higher shear stress values than the raw flour slurry due mainly to protein denaturation and starch gelatinization occurring during heat treatment. Slurries of flours T-2 and T-3 showed the highest shear stress values (Fig. 2). The apparent viscosities of raw chickpea flour and precooked flour slurries of different concentration are reported in Table 4. All the samples, even the most concentrated ones (10%), had very low viscosities with a maximum of 12 centipoises for the 10% flour T-3 slurry. An ANOVA and Tukey's Test was performed on the results of Table 4. For the 1% and 2% concentrations there was no significant difference among different samples of the same concentration. For the 5% concentration there was a significant difference ($p \leq 0.05$) between raw flour and flour T-1, but no difference between flours T-2 and T-3. For the 10% concentration the four samples were all significantly different although the difference between flours T-2 and T-3 was small. For all the samples there was no significant difference ($p \leq 0.01$) between the 1% and the 2% concentrations. For the raw chickpea flour there was also no significant difference between the 2% and 5% concentrations.

SUMMARY & CONCLUSIONS

DRUM-DRIED chickpea flours with different flavor and functional characteristics can be prepared by different methods. Acidification of the cooking or the soaking water resulted in precooked flours with reduced beany flavor. The different processes did not have a great effect on the proximate composition of the precooked flours which were all instantly dispersible in water at room temperature. The acid-treated flours showed higher oil absorption capacity but lower bulk density than the $NaHCO_3$-treated flour. All the precooked flours had the same water absorption capacity. Processing reduced the nitrogen solubility of the precooked flours but the acid treated flours showed a slightly higher NSI than the $NaHCO_3$-treated flour. Raw chickpea flour was the only whippable product and manifested good foaming qualities. Defatting increased whippability of the raw flour and its foam stability. All the precooked flours could be considered practically unwhippable. They were able to form a strong gel at a minimum concentration of 14%. Precooked flour slurries of different concentrations manifested very low viscosities even at 10% concentration. However, the acid-treated flour slurries showed higher viscosities than the $NaHCO_3$-treated flour slurry with the flour from acid-treated seeds being the most viscous.

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Minerals and Protein in Four Hard Red Winter Wheat Varieties and Fractions Derived Therefrom

F. Y. ISKANDER and M. M. MORAD

ABSTRACT

Whole grain wheat from hard red winter wheat cultivars (Scout 66, Vona, TAM101, and TAM105) and six ground fractions separated according to particle size were analyzed for 25 elements by instrumental neutron activation analysis. The apparent concentrations established in the grain for As, Ce, Co, Cr, Cs, Eu, Hf, La, Sb, Sc, Sr, Th and U were below the detection limit. However, for the four varieties, average concentrations in the whole grain (μg/g on a dry weight basis) were as follows: Br 6.55; Ca 609; Cl 651; Fe 43.3; K 4090; Mg 1460; Mn 38.5; Na 17.7; Rb 2.26; Se 0.56; and Zn 20.7. Mercury was also detected in cultivar TAM101 at a concentration of 97 ng/g. The correlation between the elements appeared to depend on the particle size distribution.

INTRODUCTION

WHEAT GRAIN and its milled fractions are known to be important sources of minerals for man and livestock. The literature provides good index on how minerals can be determined in wheat, bran and flours. Lorenz et al. (1980) reported Fe, Mg, Ca and Zn concentrations in 63 samples of wheat flour. They used atomic absorption for Fe, Mg, Ca, Zn and colorimetric methods for Fe. Garcia et al. (1972) determined the mineral constituents (K, Mg, Ca, Na, Fe, Cu, Mn, and Zn) in corn and wheat germ by wet-ashing the samples followed by atomic absorption. Phosphorus was measured colorimetrically. Peterson et al. (1983) utilized energy-dispersive X-ray spectrometry to determine Mg, P, S, Cl, K, Ca, Mn, Fe, Cu, and Zn. The concentration of P, K, Mg, Ca, Zn, Fe, Mn, and Cu was reported by Pomeranz and Dikeman (1983) in samples of hard red winter wheat flour using atomic absorption and colorimetric methods. The concentrations of Ca, P, K, Na, Mg, Zn, Fe, Mn, Cu, Se, Br, Sr, Al, Ba, and Co were determined in flours and millfeeds. Except for Co and Se, the analytical technique used was emission spectroscopy (Waggle et al., 1967). Nadkarni and Ehmann (1971) determined Hg, Fe, Zn, Br, Ag, Cr, As, Se, La, Co, Sc, Sb, Cs, and Eu in four wheat flour samples by neutron activation analysis (NAA).

Measurement of elements by atomic absorption or colorimetric techniques usually requires the minerals to be in solution. Thus, samples were either (a) ashed with concentrated acid, (b) dry ashed then dissolved in acid, or (c) pressure digested with acid. Consequently, there is a potential for contamination of the sample with impurities present in the reagent used; hence, a blank correction is required. Neutron activation analysis, on the other hand, requires no chemical pre-treatment of the sample which minimizes possible contamination and makes it an attractive technique to measure trace and minor elements in a complex matrix such as plant materials. Other advantages of NAA include multielement determination capabilities and low detection limits for certain elements.

Author Iskander is with the Nuclear Engineering Teaching Laboratory, Dept. of Mechanical Engineering, The Univ. of Texas at Austin, Austin, TX 78712. Author Morad is with the Cereal Quality Laboratory, Dept. of Soil & Crop Sciences, Texas A&M Univ., College Station, TX 77843-2474.

protein content in wheat was reported to be influenced by variety, soil type and treatment (El-Gendy et al., 1957). Consistent correlations were observed between protein and Zn and between protein and Ca (Pomeranz and Dikeman, 1983).

The purposes of this study were to determine the concentration of 25 trace and minor elements in whole grain from four hard red winter wheat cultivars, and to investigate the distribution of these elements in six ground fractions ranging from +30 through −150 mesh. Protein and *in vitro* protein digestibility for the whole grain and its fractions were also determined.

MATERIALS & METHODS

Sample preparation

Four hard red winter wheat cultivars (Scout 66, Vona, TAM101 and TAM105) grown in Chilicothe, Texas in 1982 were tested. Grain samples (100g) were tempered to 15% moisture for 18 hours before milling. A Quadrumat Junior Mill (C.W. Brabender Instruments, Inc., South Hackensack, NJ) was used to grind the whole wheat. Without sifting, the ground whole wheat was collected and used for particle size separation. Each ground sample was placed on top of a stack of five U.S. Standard Testing Sieves in the following order: #30, #40, #70, #100, and #150. Three rubber bouncers were inserted into each sieve, and a stack of sieves was placed on a Ro-Tap sifter for exactly 3 min. The first five resultant fractions were on each sieve (F-1 to F-5); whereas, the sixth fraction (F-6) was the material that passed through sieve #150. The six fractions were then collected and weighed.

Neutron activation analysis

Approximately 0.3–0.5g sample of each of the six fractions was thermally sealed into a 2/5-dram high-purity polyethylene vial. Each polyethylene vial was encapsulated into a 2-dram vial. Whole grain samples were treated similarly after being ground and passed through 1 mm screen. Standard materials used were also encapsulated in the same manner and included the following National Bureau of Standards standard reference materials: Citrus Leaves (SRM 1572), Wheat Flour (SRM 1567) and Tomato Leaves (SRM 1573). In addition, very high purity chemical salts (>99.99%) were used to prepared standards for those elements not certified in the aforementioned SRMs but determined in this study.

The samples and standard materials were irradiated in the sealed vials for the specified period of time for individual elements at a neutron flux of 2×10^{12} n $cm^{-2}sec^{-1}$ in the TRIGA Mark I reactor at The University of Texas at Austin. The associated irradiation period, decay time and counting time for each element are presented in Fig. 1. After a specific decay period, the gamma-rays emitted were measured with a high purity germanium detector (EG&G ORTEC) in a fixed geometry configuration. The detector specifications were 1.9 KeV FWHM for the 1.33 MeV ^{60}Co line, with a 20% relative efficiency. Counting acqusition of data reduction were controlled by a local computer (EG&G ORTEC). Details on the NAA procedures and instruments used were given elsewhere (Iskander, 1985; Iskander et al. 1986).

Nitrogen was determined by the modified micro-Kjeldahl procedure (Technicon, 1977). Protein was Nx5.7. *In vitro* protein digestibility via pronase hydrolysis was determined using the method of Hahn et al. (1982). Data was statistically analyzed using one-way analysis of variance and Duncan's New Multiple Range Test (Steel and Torrie, 1960).

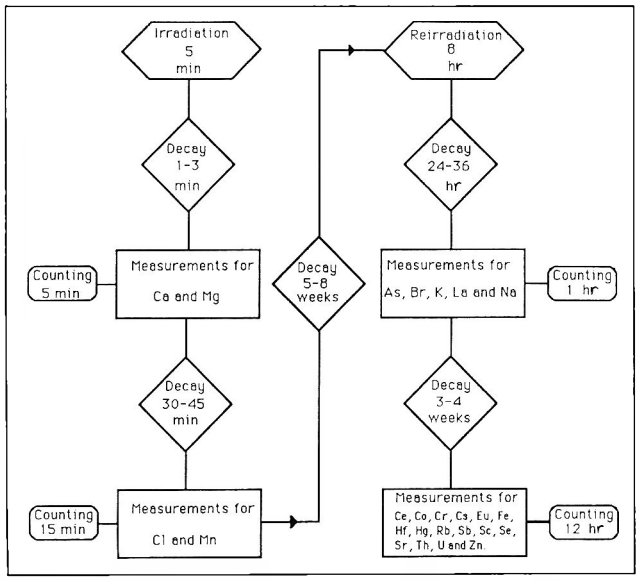

Fig. 1—Flow chart for the measurement of 25 elements by neutron activation analysis.

RESULTS & DISCUSSION

THE CONCENTRATION LEVELS of 25 elements in the whole grain derived from four wheat cultivars are shown in Table 1. However, the concentration of As, Ce, Co, Cr, Cs, Eu, Hf, Hg (except for cultivar TAM101), La, Sb, Sc, Sr, Th, and U were below the detection limit under the experimental condition. Levels of some elements found in hard red winter wheat cultivars reported in the literature are also included in Table 1. Our data for Fe, K, Mg, Mn, and Zn were below the average values reported by Dikeman et al. (1982), and Zook et al. (1970), with the exception of Ca. The data reported in this study was the arithmetic mean for duplicate analyses of samples based on a dry weight basis. For the four cultivars, the relative standard deviation of element levels was less than 10% except for Br, Sc and Se where it reached 15%. However, in case of elements listed below the detection limit (e.g., Ce), only the highest value of the duplicates was reported.

The percent each wheat fraction represents of the total whole grain is given in Table 2. The differences in the amount of the six fractions between the four cultivars were due to particle size distribution and probably morphological differences in wheat kernels.

The concentration levels of Br, Ca, Cl, Fe, K, Mg, Mn, Na, Rb, Se, and Zn in the six fractions of the four varieties are also shown in Table 2. The concentrations of each individual element were similar for the same fractions represented. Concentrations of Ca and Fe in F-1 were about fourfold their concentration in F-3 to F-6, and Mg in F-1 more than eightfold. Hard wheat flours contained, on the average, 139 μg/g Fe; 290 μg/g Mg; and 9.88 μ g/g Zn (Lorenze et al., 1980). These values are below the concentration detected for milling fractions in this investigation. The concentrations of Fe and Zn in the milling fraction were higher than those determined in commercial flour, probably because wheat germ and pericarp were included (Nadkarni and Ehmann, 1971).

The amount of an individual element represented in each separated fraction depends on both the weight of the fraction (as a percent of whole grain) and the concentration of that element in the fraction. For comparative purposes, Fig. 2 shows the calculated weight distribution of Br, Ca, Cl, Fe, K, Mg, Mn, Na, Rb, Se, and Zn in the six fractions for the four wheat cultivars. The lowest quantity of Br was found in F-2 for all the cultivars examined. The first fraction (F-1) in both Vona and TAM101 varieties contained more than 50% Ca; 60% K; 70% Mg; 50% Na; and 50% Rb of the combined fractions.

The correlation coefficient (r) (Table 3) between the 11 element concentrations in a specific fraction of the four cultivars, as well as between the elements in the whole grain were calculated. No distinct pattern was observed in the six fractions and whole grain. For example, while Fe concentration was highly correlated to Mg ($r = 0.965$) in whole grain, no such correlation was observed in milled fractions F-1 to F-6 (r values were -0.090; 0.593; -0.712; 0.385; 0.681 and -0.072 respectively). However, a high positive correlation ($1.000 > r > 0.900$), or a high negative correlation ($-1.000 < r < -0.900$) was observed between elements in every fraction and in whole grain as shown in Table 3.

Protein and *in vitro* protein digestibility measurements of the four wheat cultivars and its fractions are shown in Table 4. The average protein for the four cultivars was 15.1%. The higher *in vitro* protein digestibility values for whole grain

Table 1—Concentration levels of 25 elements in whole grain from four hard red winter wheat cultivars determined by neutron activation analysis (μg/g on dry weight basis)

Element	Scout 66	Vona	TAM 101	TAM 105	A[a]	B[b]	C[c]
AS	<0.1	<0.1	<0.2	<0.2			
Br	5.74	6.51	7.90	5.27			
Ca	610	574	585	666	487	551	
Ce	<0.09	<0.1	<0.1	<0.1			
Cl	573	646	732	653			
Co	<0.05	<0.02	<0.05	<0.05			
Cr	<0.1	<0.1	<0.2	<0.2			
Cs	<0.01	<0.01	<0.009	<0.009			
Eu	<0.001	<0.002	<0.002	<0.002			
Fe	43.3	43.6	41.1	45.1	49.7	40.2	
Hf	<0.006	<0.009	<0.01	<0.009			
Hg	<0.05	<0.06	<0.097	<0.06			
K	3530	4630	4090	4110	4560	4960	
La	<0.02	<0.02	<0.02	<0.02			
Mg	1460	1450	1400	1520	1460	1550	1800
Mn	36.0	42.0	39.0	37.0	56.0	47.4	37.9
Na	17.1	21.6	16.7	15.2			
Rb	2.30	2.15	2.13	2.45			
Sb	<0.06	<0.07	<0.07	<0.05			
Sc	<0.001	<0.001	<0.001	<0.001			
Se	0.67	0.58	0.48	0.62			
Sr	<6	<7	<7	<7			
Th	<0.005	<0.009	<0.01	<0.01			
U	<0.01	<0.06	<0.1	<0.1			
Zn	21.6	19.6	21.2	20.2	27.9	25.1	24.0

[a] Average for ten varieties cultivated in 1973. Data from Dikeman et al. (1982).
[b] Average for nine varieties cultivated in 1979. Data from Dikeman et al. (1982).
[c] Hard red winter wheat. Data from Zook et al. (1970).

Table 2—Concentration of 11 elements in the six milling fractions of four hard red winter wheat cultivars (μg/g on dry weight basis)

Cultivar	Fraction I.D.	Fraction wt (%)[a]	Elements measured										
			Br	Ca	Cl	Fe	K	Mg	Mn	Na	Rb	Se	Zn
Scout 66	F-1	9.06	12.4	1650	527	153	13500	6120	167	48.7	5.50	0.92	65.5
	F-2	4.54	11.7	1360	483	157	10400	4120	151	40.9	5.94	0.80	78.8
	F-3	22.6	13.2	327	606	20.4	2190	842	20.1	10.9	1.52	0.49	14.6
	F-4	15.7	13.9	318	860	22.4	1930	443	10.9	10.4	1.62	0.49	11.1
	F-5	23.6	15.3	379	810	29.1	1740	509	8.61	17.6	1.39	0.55	9.91
	F-6	24.5	19.5	333	813	37.1	1690	396	6.41	17.5	1.33	0.57	12.5
Vona	F-1	26.1	10.4	1340	759	148	11300	4910	147	52.4	4.99	0.48	81.1
	F-2	4.00	8.85	873	723	109	7160	2880	109	30.5	3.77	0.41	69.6
	F-3	19.9	9.88	349	826	46.4	2490	577	15.8	13.7	1.45	0.34	15.8
	F-4	15.0	8.90	281	719	32.1	1820	355	5.38	10.1	<1	0.21	6.82
	F-5	21.5	9.36	256	792	<17	1830	353	5.14	10.9	1.36	0.27	7.28
	F-6	13.5	8.02	334	689	46.4	1840	387	4.48	19.5	2.05	0.48	13.9
TAM 101	F-1	21.8	10.1	1320	579	137	12500	5070	154	41.1	6.31	0.56	77.5
	F-2	4.00	10.0	993	550	91.2	8240	3220	129	28.7	3.61	0.57	65.2
	F-3	26.0	11.3	343	821	31.7	2250	560	18.6	10.8	1.38	0.49	12.8
	F-4	15.6	12.5	290	813	<10	1730	382	7.18	9.65	1.54	0.29	6.43
	F-5	12.0	12.3	293	847	23.7	1770	415	6.66	9.69	<1	0.43	7.12
	F-6	20.6	12.9	306	785	22.7	1730	391	6.94	12.4	1.54	0.43	8.61
TAM 105	F-1	13.5	10.7	1350	559	115	13000	5700	179	43.7	5.79	0.68	58.6
	F-2	5.90	9.92	1170	623	112	10400	4200	183	37.1	4.54	0.56	66.6
	F-3	23.4	11.3	429	740	28.8	3000	888	34.2	13.2	1.75	0.41	18.9
	F-4	17.6	11.3	238	735	<9	1540	270	8.57	9.51	1.00	0.35	6.15
	F-5	17.1	11.3	310	712	25.3	1510	335	8.03	10.9	0.99	0.35	10.2
	F-6	22.5	12.5	274	752	34.3	1720	515	7.93	14.0	1.22	0.38	10.2

[a] Fraction % of total weight. Fractions F-1 through F-6 represent +30, +40, +70, +100, +150, and −150 mesh particle sizes, respectively, for all varieties.

Table 3—Correlation between element concentration in the whole grain and in the six milling fractions of the four hard red winter wheat cultivars

Whole grain	HPC[a]	Mg/Fe	Rb/Ca	Mn/K	Rb/Mg				
	HNC	Fe/Br	Se/Cl						
	NC	Fe/k	Mg/k	Zn/Na					
F-1	HPC	Ca/Br	Mg/K	Ca/Se	Br/Se				
	HNC	Cl/K	Rb/Na	Zn/Mn					
	NC	Mg/Fe	Mg/Na	Rb/Mg	Se/Na	Se/Rb			
F-2	HPC	Ca/Br	Ca/k	Mg/K	Mg/Ca	Mn/K	Na/Ca	Rb/Fe	
		Rb/Ca	Rb/Se	Mn/Mg	Rb/Na				
	HNC	Br/Cl	Se/Cl						
	NC	Zn/Mn							
F-3	HPC	Ca/K	Mn/Ca	Zn/K	Rb/Mn	Zn/Rb			
	HNC	Cl/Br	Se/Na						
	NC	Rb/Fe							
F-4	HPC	Cl/Br	Ca/K	Mg/Ca	Mg/K	Na/K		Rb/Cl	Rb/Ca
		Rb/Mg	Se/Mn						
	NC	Rb/Fe							
F-5	NPC	Ca/Br	Fe/Ca	Fe/Br	Mg/Br	Mn/Ca	Mn/Fe	Se/Br	Se/Ca
		Se/Mg							
	NC	Cl/Ca	Fe/Cl	Br/K	Na/K	Rb/Fe	Se/K	Zn/Rb	
F-6	HPC	Cl/Br	Na/Fe	Rb/K	Zn/Fe	Zn/Na			
	HNC	Cl/K	Rb/Mn						
	NC	Cl/Ca	Mg/Fe	Mg/Cl	Mg/Br	Se/K			

[a] HPC: High positive correlation 1>r>0.9. HNC: High negative correlation (−1)<r<(−0.9). NC: No correlation 0.1>r>(−0.1).

Table 4—Protein content and in vitro *protein digestibility for four hard red winter wheat cultivars and its milling fractions*[a]

	Scout 66		Vona		TAM 101		TAM 105	
	Pr[b] %	Di[b] %	Pr %	Di %	Pr %	Di %	Pr %	Di %
Whole	15.6 c	62.3 c	15.0 c	69.3 b	14.6 a,b	69.2 b	15.2 c	73.8 b,c
F-1	20.5 a	58.3 d	18.1 a	58.7 d	15.8 b	65.0 c	18.0 b	68.6 c
F-2	20.4 a	58.6 d	17.7 b	63.6 c	18.4 a	54.4 d	18.9 a	70.3 c
F-3	14.6 d	67.1 b	12.9 e	75.4 a	12.9 d	79.8 a	13.5 d	90.9 a
F-4	14.4 d	70.2 a	13.2 e	75.9 a	13.0 d	68.3 b	12.1 e	90.1 a
F-5	15.4 c	67.1 b	14.3 d	70.0 b	13.9 c	71.0 b	13.5 d	79.5 b
F-6	16.8 b	66.4 b,c	15.3 c	72.9 a,b	15.8 b	69.3 b	14.8 c	79.5 b

[a] Within each column, values followed by the same letter are not significantly different by Duncan's multiple range test (P = 0.01). CV < 5% for all means.
[b] N × 5.7. Pr: Protein (n = 2). Di: Protein digestibility, (n = 2).

(83.7%) reported by Wolzak et al. (1981), compared to 69.7% (mean value) for the four cultivars examined, may be related to differences in procedure used for determination.

For all cultivars, fraction 1 and 2 contained significantly higher protein while fractions 3 through 5 were similarly lower. Protein concentration in the whole grain and F-6 was intermediate. Jones et al. (1959) studied the separation of flour into fractions by means of air classification. They reported the coarse fractions to contain the highest protein concentration and the finest fraction to be similar to the original grain in protein content. The intermediate fractions were equally low in protein concentration. Wheat germ and aleurone layer contained higher protein concentration compared to the endosperm and they usually separated in the coarse fraction during wheat milling

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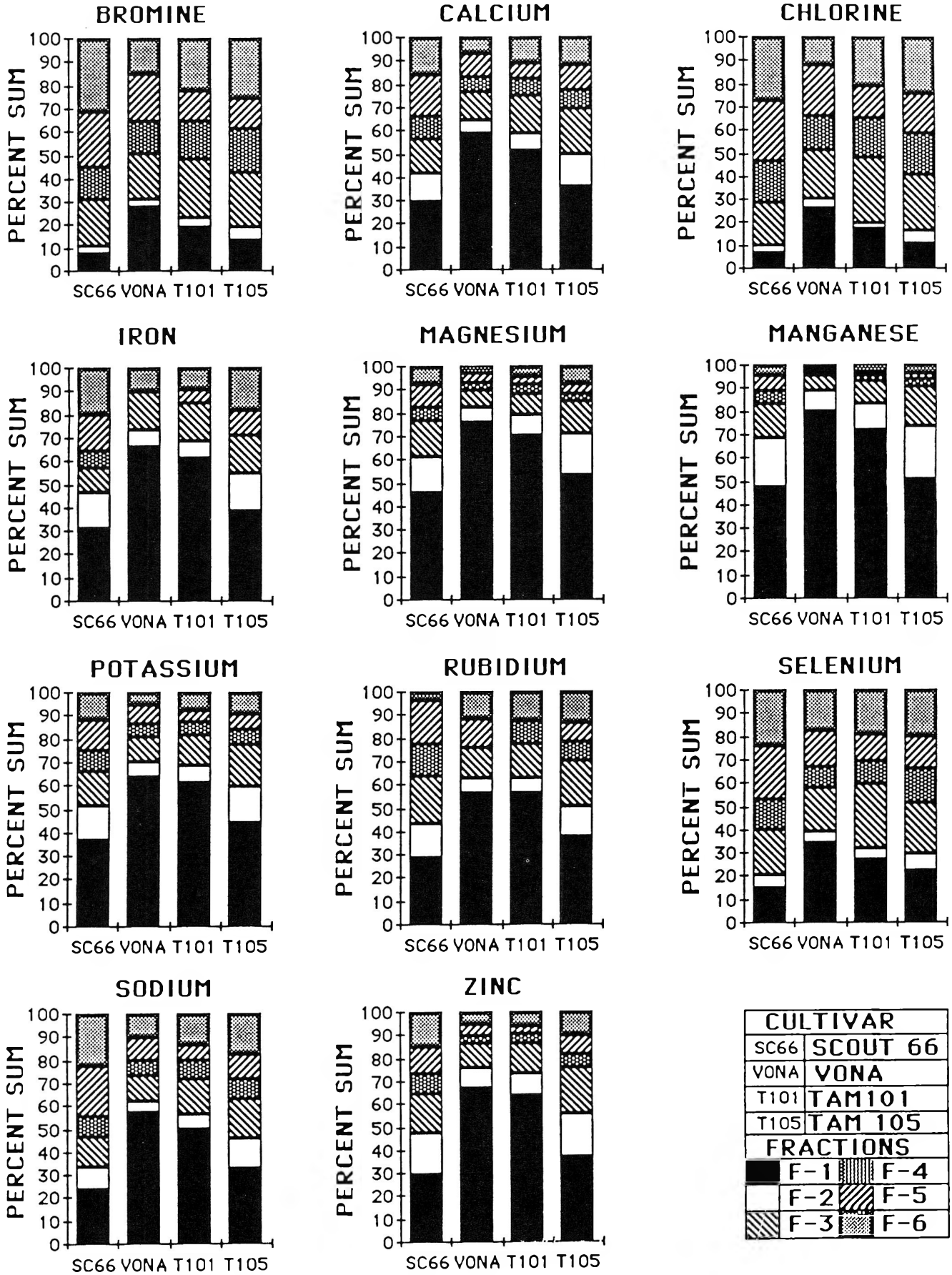

Fig. 2—Distribution of Br, Ca, Cl, Fe, Mg, Mn, K, Rb, Se, Na and Zn in the six fractions of the four hard red winter wheat varieties.

(Pomeranz, 1971). The *in vitro* protein digestibility of the coarse fractions were lower (Table 4), while those of the finer fractions (F-3 to F-6) were higher. This can probably be explained by the presence of higher levels of fiber in the larger particle

size fractions (mainly seed coats of higher phytic acid concentration).

The correlation coefficients (r) between protein content and individual element concentration, and between *in vitro* protein digestibility values and elements concentration were calculated. No definitive pattern was found. For example, protein and Br content were strongly (positive) correlated in F-1, F-2, and F-3 fractions (r = 0.910; 0.974, and 0.900 respectively). Such correlations did not exist in fractions F-4, F-5, and F-6 (r = 0.462, 0.667, and 0.774, respectively). For whole grain the corresponding value of r was −0.924.

CONCLUSION

OF THE 25 ELEMENTS studied in the whole grain by neutron activation analysis, 11 elements were both positively identified and measured in four hard red wheat cultivars. Each of the four cultivars was ground and fractionated into six fractions according to particle size. The concentrations of nutritionally important elements (Ca, Fe, Mg, Mn, and Zn) were the highest in the coarse fraction (material retained by sieve #30) while the lowest concentration were in the finest fraction (material through sieve #150). The correlation between protein and element content, or among some of the elements themselves depends on particle size distribution (i.e., different correlation for different milling fraction).

This study may be useful to wheat growers and millers in understanding the effect of milling procedures and wheat refining on mineral content of wheat and flour used for baking purposes. The study may also help in the assessment of mineral fortification.

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Inhibitory Potency of Plant Antinutrients towards the In Vitro Digestibility of Buckwheat Protein

K. IKEDA, M. OKU, T. KUSANO and K. YASUMOTO

ABSTRACT

The inhibitory potency of dietary fiber sources, tannins, phytate, and a protein protease inhibitor on the *in vitro* pepsin-pancreatin digestibility of protein was compared. The protein inhibitor exhibited the highest inhibitory capacity among the substances examined; and phytate the lowest. Our findings suggest that the inhibition of the digestion of dietary sources of plant proteins by such factors as fiber and tannins, in addition to the protein protease inhibitors, may also be taken into account.

INTRODUCTION

LEGUMES AND CEREALS constitute a staple part of dietary protein of large segments of the world's population. While the seeds contain moderate amounts of protein, they also contain a number of constituents which adversely affect the utilization of the protein present (Liener and Kakade, 1980). Protein protease inhibitors have been shown to be widely found in the plant kingdom (Liener and Kakade, 1980). We have reported evidence that buckwheat (*Fagopyrum esculentum* Moench) seed, an important source of dietary protein in some areas of the world, contains a protein trypsin inhibitor (Ikeda and Kusano, 1978, 1983a). Among the many deleterious factors present in edible seeds, protein protease inhibitors have been most extensively investigated. Studies with laboratory animals have shown that protease inhibitors may inhibit growth, reduce digestibility, and cause pancreatic hypertrophy (Liener and Kakade, 1980). But, the correlation between the nutritive value of edible seeds and the content of the protease inhibitors present is the subject of much controversy.

There are some other antinutritional factors adversely affecting the biological availability of proteins in many edible seeds. The seeds of many legumes have long been known to contain proteins which agglutinate erythrocytes (Boyd, 1963). The hemagglutinins (lectins) may reduce the intestinal absorption of essential nutrients through their combining with the absorptive cells lining the intestinal wall (Jaffé, 1980). Tannins are known to impair utilization of proteins in human and animal diets by binding with and coagulating protein (Butler *et al.*, 1984; Reddy *et al.*, 1985). Growth retardation has been observed in animals fed diets containing tannins (Joslyn and Glick, 1969). Phytate, a common constituent of plant tissues, has been shown to have an inhibitory action against a proteolytic enzyme (Kratzer, 1965; Kanaya *et al.*, 1976; Knuckles *et al.*, 1985).

Current evidence suggests that dietary fiber can express an inhibitory effect on the assimilation of certain essential nutrients from the gastrointestinal tract. Many investigators have observed that a significant increase in fecal nitrogen excretion occurs on high-fiber diets (Spiller *et al.*, 1975; Southgate *et al.*, 1976; Kelsay *et al.*, 1978; Calloway and Kretsch, 1978; Harmuth-Hoene and Schwerdtfeger, 1979; Shah *et al.*, 1982). The elevation of the activities of the pancreatic digestive enzymes, including proteolytic enzymes, has been reported with animals fed high-fiber diets (Sheard and Schneeman, 1980; Schneeman *et al.*, 1982). We have reported evidence that several dietary fiber sources directly exert an inhibitory effect on the *in vitro* activities of proteolytic enzymes (Ikeda and Kusano, 1983b). These studies suggest that dietary fiber is not innocuous in the alimentary tract, but may reduce availability of dietary proteins.

A number of antinutrients may reduce the bioavailability of plant seed proteins through their inhibitory activities towards the digestion or absorption. However, the correlation between the nutritive value of edible seeds and the total level of their inherent antinutrients is not clear. The question of what components in edible seeds produce most significant inhibition also remains unanswered. The present study was designed to estimate the relative inhibitory potency towards the *in vitro* digestibility of a specific protein, *i.e.*, the globulin of buckwheat seed, of dietary fiber, tannins, phytate, and the endogenous protein inhibitor.

Authors Ikeda, Oku, and Kusano are with the Faculty of Nutrition, Kobe-Gakuin Univ., Ikawadani-cho, Nishi-ku, Kobe 673, Japan. Author Yasumoto is with the Research Institute for Food Science, Kyoto Univ., Uji, Kyoto 611, Japan.

MATERIALS & METHODS

Materials

The globulin of buckwheat (*Fagopyrum esculentum* Moench) seeds was used as a protein sample in this study. Buckwheat seeds, harvested in November, 1983, in Japan, were obtained and stored at 4°C until used. The globulin was isolated from the seeds according to the procedure of Javornik and Kreft (1984). The protein (N × 6.25) content of the globulin, as determined by the micro-Kjeldahl method (AOAC, 1980), was 94.6 ± 5.0% (means ± S.D., n=4) on a dry weight basis. The globulin preparation contained 10.6 ± 0.3 mg phosphorus (means ± S.D., n=4) per 100g solid on a dry weight basis, but no dietary fiber, as assayed by the gravimetric, enzymatic method (Asp *et al.*, 1983), was found in the protein preparation. The endogenous, protein protease inhibitor of buckwheat seeds was prepared by the chromatographic procedure on Sephadex G-75 (Ikeda and Kusano, 1978). Nine different sources of dietary fiber were obtained from the following companies: guar gum, pectin from citrus fruits, tragacanth gum, and xylan from oat spelts, from Sigma Chemicals Co.; inulin, pectic acid, and sodium alginate, from Nakarai Chemicals, Ltd. (Japan); agar-agar, from Difco Laboratories; and cellulose powder, from M. Nagel Co. The neutral detergent fiber of buckwheat flour was prepared according to the procedure of Robertson and Van Soest (1977). Tannic acid was obtained from E. Merck, Darmstadt; and catechin, from Nakarai Chemicals, Ltd. Enzymes used in this study were obtained from the following companies: trypsin (EC 3.4.21.4; 2 × crystalline, from bovine pancreas, 12,000 BAEE units/mg protein) and pepsin (EC 3.4.23.1; 2 × crystalline, from porcine stomach mucosa), from Sigma Chemicals Co.; and pancreatin NF, from Difco Laboratories. Benzoyl-D,L-arginine *p*-nitroanilide (BApNA) and benzoyl-L-tyrosine *p*-nitroanilide (BTpNA) were obtained from Nakarai Chemicals, Ltd. Sephadex G-75 was obtained from Pharmacia Fine Chemicals; and Toyopearl HW-50, from Toyo Soda MFG, Co. (Japan). All other chemicals were analytical grade.

In vitro proteolytic digestion

In vitro proteolytic digestion was conducted to determine the effect of the presence of selected antinutrients (protein inhibitor, dietary fiber sources, tannins, and phytate) on the concentration of free peptides released upon protein hydrolysis. The digestion was performed with the buckwheat globulin as the reference protein using the procedure

Table 1—Inhibition of trypsin activity by sources of dietary fiber, tannins, phytate, and buckwheat protein inhibitor[a]

Substances tested	Amounts of the inhibitors required for half inhibition (mg/mg trypsin)
Agar-agar	633 ± 10
Pectin	614 ± 88
Sodium alginate	454 ± 40
Xylan	225 ± 80
Catechin	no effect
Tannic acid	23.0 ± 7.2
Sodium phytate	no effect
Buckwheat protein inhibitor	0.89 ± 0.14

[a] Values are means ± S.D. (n=5).

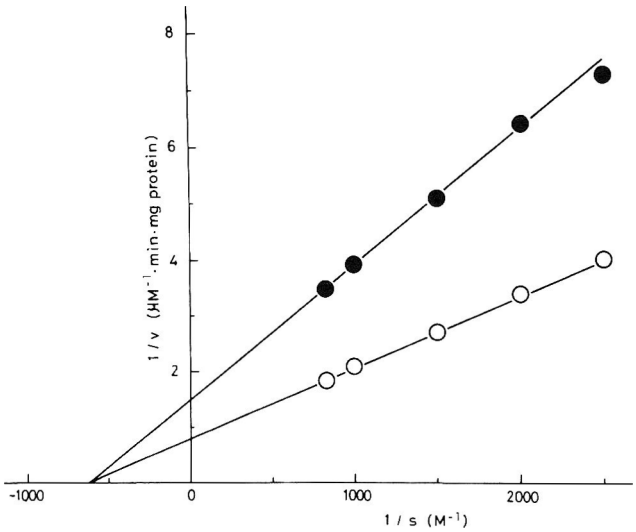

Fig. 1—Noncompetitive inhibition of trypsin activity by tannic acid. The control reaction mixture contained 1.0 nmol of trypsin, the indicated concentration of BApNA, 980 μmol of Tris-HCl buffer (pH 8.2), and 150 μmol of $CaCl_2$ in a total volume of 4.0 mL. ○———○ without tannic acid; and ●———● with tannic acid (900 μg).

Table 2—Effects of various dietary fiber sources on the peptic and pancreatic digestion of buckwheat globulin

Dietary fiber sources	Digestibility (%) Weight added of fiber: 10-fold wt to protein	20-fold wt to protein
None	34.9 ± 0.5[ab]	34.9 ± 0.5[a]
Agar-agar	30.2 ± 0.4[d]	24.0 ± 0.5[e]
Buckwheat neutral detergent fiber	31.1 ± 0.5[cd]	31.5 ± 0.6[b]
Cellulose powder	35.0 ± 0.1[a]	32.9 ± 0.9[ab]
Guar gum	30.3 ± 0.8[cd]	28.0 ± 1.4[cd]
Inulin	32.7 ± 0.9[bcd]	33.9 ± 0.5[ab]
Pectin acid	33.1 ± 0.9[ac]	32.1 ± 0.9[b]
Pectin	29.7 ± 0.9[d]	25.7 ± 0.8[de]
Sodium alginate	32.8 ± 0.4[c]	27.7 ± 1.2[cd]
Tragacanth gum	32.8 ± 1.3[acd]	28.4 ± 1.0[cd]
Xylan	31.5 ± 0.4[cd]	28.9 ± 0.2[c]

[a-e] Values are means ± S.D. (n=3). Values within a column that do not share a common superscript are significantly different at $p<0.05$.

of Akeson and Stahmann (1964) but with a slight modification (Ikeda *et al.*, 1984a). The enzymatic assay consisted of a pH 1.0 hydrolysis for 3 hr by pepsin, followed by a pH 8.0 hydrolysis for 20 hr by pancreatin. Ten ml aliquots of a globulin solution (2.5 mg/mL) were hydrolyzed in the presence or absence of the selected antinutrients in a final volume of 25.0 mL, and the enzyme-to-protein ratio was 1:50.

Table 3—Effect of the protein inhibitor on the peptic and pancreatic digestion of buckwheat globulin

Protein inhibitor added	Peptides in the digesta (μg/25 mg protein)	Digestibility (%)
None	8736 ± 128[a]	34.9[a]
0.0125-fold wt to protein	8254 ± 99[b]	33.0[b]
0.05-fold wt to protein	6866 ± 148[c]	27.5[c]
0.1-fold wt to protein	4361 ± 29[d]	17.4[d]

[a-d] Values are means ± S.D. (n=3). Values within a column that do not share a common superscript are significantly different at $p<0.05$.

Table 4—Effects of tannins and sodium phytate on the peptic and pancreatic digestion of buckwheat globulin

Substances added	Peptides in the digesta (μg/25 mg protein) Weight added: 0.4-fold wt to protein	0.8-fold wt to protein	Digestibility (%) Weight added: 0.4-fold wt to protein	0.8-fold wt to protein
None	8736 ± 128[a]	8736 ± 128[a]	34.9[a]	34.9[a]
Catechin	8092 ± 88[b]	8107 ± 39[b]	32.4[b]	32.4[b]
Tannic acid	5738 ± 19[c]	5144 ± 74[c]	23.0[c]	20.6[c]
Sodium phytate	8661 ± 92[a]	8686 ± 180[a]	34.6[a]	34.7[a]

[a-c] Values are means ± S.D. (n=3). Values within a column that do not share a common superscript are significantly different at $p<0.05$.

The antinutritional substances examined were added to the digestion mixture prior to the addition of enzymes. Sodium azide was added to the pancreatic digestion mixture to a final concentration of 0.025% to prevent growth of microorganisms. After digestion, a 4 mL aliquot of the soluble digesta was added to a test tube containing 1 ml each of 10% sodium tungstate and 0.67N sulfuric acid (Scheffner, 1967; Kan and Shipe, 1984). It was allowed to stand for 10 min, then centrifuged at 4,000 rpm for 15 min. The supernatant obtained was assayed for peptide. Per cent protein hydrolysis was calculated from the ratio of the content of free peptides released upon the digestion to the original content of the globulin added to the assay mixture prior to digestion.

Another aliquot of the soluble digesta obtained on the pepsin and pancreatin digestion was applied on a Toyopearl HW-50 column (36 × 1.60 cm i.d.), which was previously equilibrated with 0.1M Tris HCl buffer (pH 8.0).

Assay of enzymatic and inhibitory activities

The activity of trypsin towards BApNA as the substrate was determined by measuring the amount of *p*-nitroaniline liberated from the substrate from its spectrophotometer reading at 410 nm as described by Erlanger *et al* (1961) but with a slight modification. The enzymatic assay consisted of 1.0 nmol trypsin, 4.0 μmol BApNA, 980 μmol Tris-HCl buffer (pH 8.2), 150 μmol $CaCl_2$ in a total volume of 4.0 mL. The reaction was performed at 37°C for 15 min and stopped by the addition of 1.0 mL 30% acetic acid. One unit of enzyme activity is defined as the conversion of 1 μmol substrate per min. The inhibitory activity of the selected enzyme inhibitors against trypsin was determined from the residual enzymatic activity after pre-incubation of the inhibitors with the enzyme for 10 min at 37°C. Amounts of the inhibitors required for half inhibition were estimated with varying the content of the inhibitors added to the assay mixture. The activity of chymotrypsin towards BTpNA as the substrate was determined according to the procedure described previously (Ikeda and Kusano, 1978).

Other analyses

Peptide content was determined colorimetrically with 2,4,6-trinitrobenzenesulfonic acid (Goldfarb, 1966). The amount of peptide was expressed as L-leucyl-glycine equivalent. Protein content (N × 6.25) was determined by the micro-Kjeldahl method (AOAC, 1980). The distribution of protein in column effluents was determined by A_{280} measurements. Phosphorus was assayed by the procedure of Fiske and Subbarow (1925).

Data were subjected to analysis of variance and the significance of means was tested by Duncan's method (Steel and Torrie, 1980).

Table 5—Inhibitory potency of dietary fiber sources, tannins and protein inhibitor towards the digestion of buckwheat globulin

Substances tested	Inhibitory potency towards digestion (units/mg, × 10^{-3})
Agar-agar	2.04 ± 0.21[d]
Guar gum	1.63 ± 0.39[ef]
Pectic acid	0.66 ± 0.27[h]
Pectin	1.98 ± 0.29[de]
Sodium alginate	1.16 ± 0.37[fg]
Tragacanth gum	1.09 ± 0.41[gh]
Xylan	1.30 ± 0.15[fg]
Catechin	19.2 ± 7.6[c]
Tannic acid	95.8 ± 26.4[b]
Protein inhibitor	649 ± 63[a]

[a-h] One unit of inhibitor is defined as the inhibition of the conversion of 1 mg the globulin into soluble peptide under the digestion conditions employed. Values were calculated from the digestibility data of Tables 2 to 4. Values within a column that do not share a common superscript are significantly different at $p<0.05$.

RESULTS & DISCUSSION

Inhibition of trypsin by antinutritional substances

The inhibitory capacities of dietary fiber sources, tannins, phytate, and the protein trypsin inhibitor against the hydrolytic activity of trypsin towards BApNA are shown in Table 1. The protein inhibitor exhibited the highest inhibitory capacity among the substances examined. The dietary fiber sources had relatively low inhibitory capacity against trypsin. A slight, but not significant ($p<0.05$), increase in the trypsin-inhibitory activity of all the dietary fiber sources examined was found when the fiber had been added to the substrate prior to the addition of the enzyme (data not shown). We have recently shown that the inhibition of trypsin activity by several dietary fiber sources was found not only with BApNA but also with the protein substrate, *i.e.*, casein and hemoglobin, and that the inhibition conformed to a parabolic noncompetitive form (Ikeda and Kusano, 1983b), suggesting that the enzyme inhibition by the dietary fiber sources may come mainly from their interaction with the substrate. In fact, our previous studies have indicated that several sources of dietary fiber could combine with proteins or peptides under slightly alkaline conditions (Ikeda and Kusano, 1983b; Ikeda *et al.*, 1984b). On the other hand, Acton *et al.* (1982) suggested that dietary fiber may reduce protein digestibility through ionic interaction, matrix restriction, and modification of filtration characteristics by the fiber.

Tannic acid exerted a relatively high inhibitory effect on the activity of trypsin (Table 1). Kinetic analyses showed that tannic acid was a noncompetitive inhibitor against trypsin (Fig. 1). In addition, the inhibitory activity of tannic acid against trypsin was approximately 1.4-fold greater when the tannic acid had been added to the substrate prior to the addition of the enzyme (data not shown). The tannins from various plant origins are known to inhibit digestive enzymes, including trypsin, through their ability to bind with proteins (Butler *et al.*, 1984; Reddy *et al.*, 1985), however the detailed mechanism involved has been not elucidated. On the other hand, no inhibition was found with catechin and with phytate under the enzymatic assay conditions employed (Table 1).

Effects of the antinutritional substances on protein digestibility

The inhibitory effects of the dietary fiber sources on the peptic and pancreatic digestion of buckwheat globulin are shown in Table 2. The pancreatin NF used contained approximately 4.2×10^{-2} BApNA units trypsin activity per mg solid on a dry weight basis; and approximately 2.8×10^{-2} BTpNA units chymotrypsin activity per mg solid on a dry weight basis. All the fiber sources examined, except for cellulose powder and inulin, significantly ($p<0.05$) lowered the protein digestibility. The neutral detergent fiber, prepared from buckwheat flour, was also found to reduce the digestibility of the endogenous

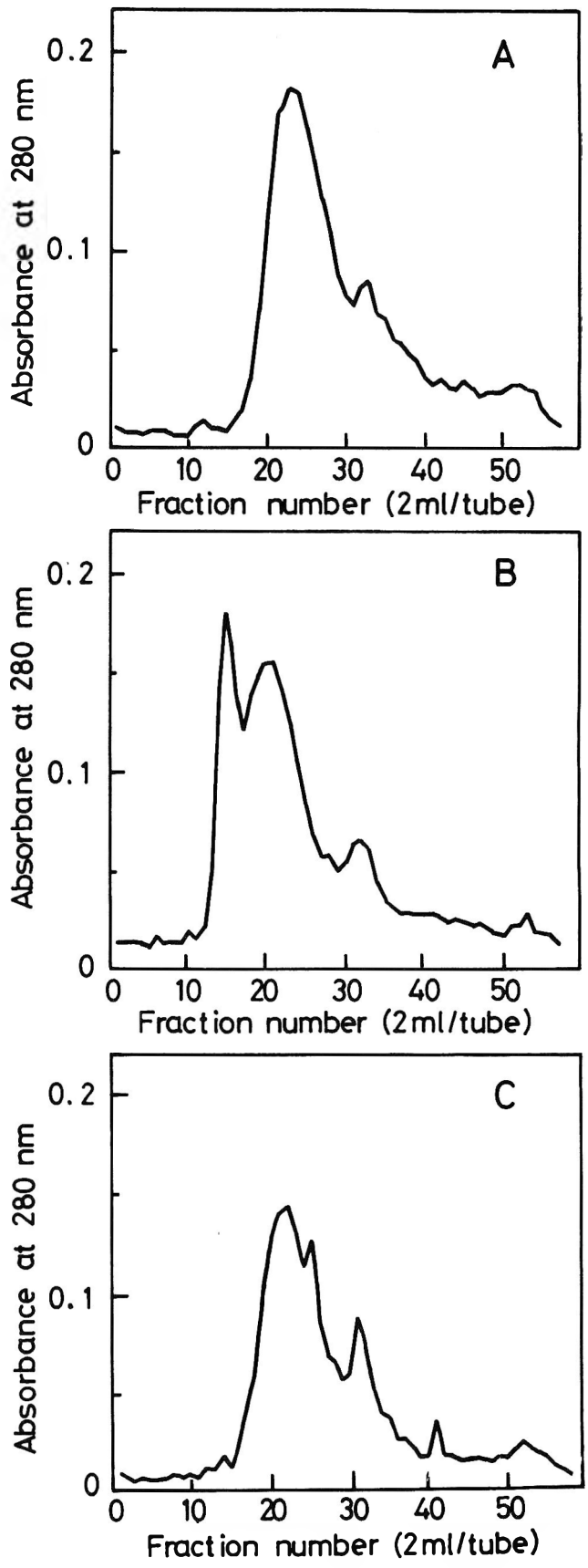

Fig. 2—Chromatographic elution profiles of the soluble digesta obtained on the in vitro digestion of buckwheat globulin in the presence or absence of antinutrients. (A) the control digesta without antinutrients; (B) the digesta with agar-agar; and (C) the digesta with the protein inhibitor.

protein. Incorporation of the protein inhibitor into the digestion mixture significantly ($p<0.05$) decreased the peptic and pancreatic digestibility of buckwheat globulin (Table 3). Tannic acid and catechin exhibited an inhibitory effect on the protein digestibility; and phytate did not exhibit any effect (Table 1). Knuckles *et al.* (1985) have observed that the inhibitory effect of phytate towards protein digestion increased with dose level but the inhibition differed with protein source.

Chromatography of the digesta in the presence or absence of the antinutritional substances on a Toyopearl HW-50 column was performed (Fig. 2). Incorporation of agar-agar into the digestion mixture led to an increase in a high-molecular-weight peptide in the digesta (Fig. 2B). A similar result was observed with pectin and xylan (data not shown). On the other hand, the protein inhibitor did not substantially alter the chromatographic elution profile as compared with the control (Fig. 2C). Tannic acid and catechin in themselves exhibited intense absorbance at 280 nm, and, therefore, these substances interfered with the direct determination of protein in column effluents monitored at 280 nm. The chromatogram, obtained from difference absorbance at 280 nm by subtraction of the enzyme-free blank, of the soluble digesta in the presence of tannic acid or catechin, indicated that these anti-nutritional substances also greatly altered the elution profile compared with the control (data not shown). Inhibitory potency of the antinutrients towards the digestion of buckwheat globulin was calculated from the digestibility data of Tables 2 to 4 (Table 5). The protein inhibitor exhibited the highest inhibitory potency among the substances examined.

Generally, the nutritive value of dietary proteins depends on the biological availability of their amino acids. Although the presence of antinutrients, as well as the relative proportions of the constituent amino acids, profoundly affects the nutritive value of edible seeds, there were few attempts to determine the correlation between the level of antinutrients in edible seeds and their overall biological impact (Thompson and Yoon, 1984; Sitren *et al.*, 1985). Legumes and cereals, on the other hand, contain 45–100 mg protein protease inhibitors per 100g seeds, representing 2.5% or more of the whole seed protein (Liener and Kakade, 1980; Ikeda and Kusano, 1978). They also contain 0–1.6% tannins (Price *et al.*, 1980), and the pigmented varieties contain 2–4% condensed tannins (Dephpande *et al.*, 1984). In addition, the plant seeds contain 9.3–23.2% dietary fiber as measured by the gravimetric, enzymatic method (James and Theander, 1981; Asp *et al.*, 1983; Frølish and Hestangen, 1983; Nyman *et al.*, 1984). In view of the observed inhibitory potency of these antinutrients against the digestion and of their levels in edible seeds, the inhibition of the digestion of dietary sources of plant proteins by such factors as fiber and tannins, in addition to the protein protease inhibitors, should also be taken into account.

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Inhibition of Polyphenoloxidase by Sulfite

L. A. SAYAVEDRA-SOTO and M. W. MONTGOMERY

ABSTRACT

When polyphenoloxidase (PPO) was exposed to sulfite prior to susbstrate addition, inhibition was irreversible. Trials to regenerate PPO activity, using extensive dialysis, column chromatography, and addition of copper salts were not successful. Increased concentrations of sulfite and pH levels less than 5 enhanced the inhibition of PPO by sulfite. At pH 4, concentrations greater than 0.04 mg/mL completely inhibited 1,000 units of PPO activity almost instantaneously. This suggested that the HSO_3^- molecule was the main component in the sulfite system inhibiting PPO. Column chromatography, extensive dialysis, and gel electrophoresis did not demonstrate $^{35}SO_2$ bound to purified pear PPO protein. Formation of extra protein bands of sulfite inhibited purified pear PPO fractions on gel electrophoresis was demonstrated. This and other evidence suggested that the major mode of direct irreversible inhibition of PPO was modification of the protein structure, with retention of its molecular unity.

INTRODUCTION

PREVENTION of undesirable browning of foods, enzymatic or nonenzymatic, has long been the concern of food scientists. Enzymatic browning is catalyzed primarily by the enzyme polyphenoloxidse (EC 1.14.18.1; PPO) and is mainly the result of the oxidation and polymerization of polyphenols. Inhibition of PPO can be achieved by heat inactivation, by the exclusion of one or all of the substrates, or by the use of additives. Sulfur dioxide (SO_2) is widely-used as an additive to avoid browning of fruits and vegetables during processing. This additive can be easily handled and generated by using sulfites, is extremely versatile (inhibits enzymatic browning as well as nonenzymatic browning; can be used as an antioxidant, bleaching agent, or antimicrobial agent), and is effective at relatively low concentration. Soluble sulfites, as well as SO_2, when dissolved in water, exist mainly as a mixture of the ionic species sulfite ($SO_3^=$) and bisulfite (HSO_3^-). The amount of each one of the ionic species varies according to the pH of the solution. At pH level of 4, HSO_3^- is at its highest concentration and at pH level of 7, both ionic species, exist approximately in the same proportion (Green, 1976).

SO_2 has been classified as a health hazard compound for asthmatics (Koeing et al., 1983) and the need for alternatives to this additive thus becomes more imperative. To suggest alternative methods other than the use of sulfite, to control enzymatic browning, the mechanism of reaction of PPO and the mode of action of sulfite have to be elucidated. Information in this respect has been relatively limited by the lack of pure PPO from fruit sources. Methodology for the isolation of pure forms of pear PPO has recently become available making possible more detailed studies (Wissemann and Montgomery, 1985).

Evidence of the mechanisms of inhibition of enzymatic browning by sulfite, such as formation of quinone-sulfite complexes and the inactivation of PPO has been found (Embs and Markakis, 1965; Haisman, 1974). This has left open for further research the direct mode of action of sulfite on the enzyme itself. The purpose of the present research was to contribute to the elucidation of the mode(s) of action of sulfite inhibition of enzymatic browning.

The authors are affiliated with the Dept. of Crop Science and Dept. of Food Science & Technology, Oregon State Univ., Corvallis, OR 97331.

MATERIALS AND METHODS

Enzyme sources

Three sources of PPO were used: a crude extract from bananas (Montgomery and Sgiarberi, 1975), a commercial mushroom preparation (Sigma product No. T-7755, lot 91F-950), and a purified form of pear PPO. Pear PPO was isolated from d'Anjou pears (*Pyrus communis* L.) grown in orchards at the Mid-Columbia Experiment Station, Hood River, OR, during the 1981 season. The pears were held for 7 months at −1°C, before being quartered and immediately dipped in liquid nitrogen. The frozen pear tissue was placed in cryovac bags and stored at −40°C until used. PPO from pears was extracted and purified using the procedure developed by Wissemann and Montgomery (1985).

Enzyme and protein determination

Activity of PPO was assayed using the method developed by Esterbauer et al. (1977) using 2-nitro-5-thiobenzoic acid (TNB). TNB was prepared by adding sodium borohydride (30 mg) to a suspension of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, 19 mg) in water (25 mL). The standard TNB assay mixture used in this research contained 1 mL 0.1M citrate-0.2M phosphate buffer (pH 5), 0.05 mL of 1.9 mM TNB and 0.05-0.1 mL of enzyme preparation to which 0.250 mL of 0.139M 4-methyl catechol in 0.01M citrate-0.02M phosphate pH 3.5 were added. Change in absorbance at 412 nm was recorded for 1–2 min using a recording spectrophotometer equipped with a chart recorder. Units of activity of the enzyme were expressed as the amount of enzyme able to transform 1 nMol of dihydric phenol to 1 nMol of quinone per minute at 20°C under the assay conditions.

The Bio-Rad protein microassay procedure was used for determining 1–20 μg of protein with bovine serum albumin as a standard. This method is based on the absorbance shift of Coomassie brilliant blue G-250 from 465 nm to 595 nm when binding to protein occurs (Bradford, 1976).

Polyacrylamide slab and SDS electrophoresis

For nondenaturing conditions, gel slabs of 7% acrylamide (0.15 cm × 14 cm × 12 in length) with 2 cm of 4.5% stacking gels with 15 wells were prepared. In most tests for PPO purity, 0.2–0.3 μg of protein per well were applied. Samples were prepared by adding 0.1 mL of 40% sucrose solution to 0.4 mL of sample containing 2–3 μg of protein per mL. Bromophenol blue (0.001%) was added as a tracking dye. Electrode buffer was 0.025M Tris-0.10M glycine, pH 8.3. Electrophoresis slabs were cooled with water to 5°C and 15 mA per slab was applied. The electrophoretic run was stopped when the tracking dye reached the bottom of the gels.

Sodium dodecyl sulfate (SDS) electrophoresis was conducted in the same manner as above except with 10% acrylamide running gels and 0.1% SDS in the stacking, running gels, and electrode buffer. PPO samples for SDS electrophoresis were prepared by boiling for 5 min, 0.4 mL of sample with 0.01g SDS, 0.05g glycerol, and 0.25 mL mercaptoethanol.

Detection of PPO isoenzymes

Immediately after stopping the electrophoretic run, the slabs were dipped and agitated gently in 15 mM catechol in 0.1M to 0.2M phosphate buffer (pH 5) and 0.05M p-phenylenediamine until bands showing PPO activity were evident (30–45 min). Then the slabs were rinsed with water and allowed to stand for 5 min with gentle agitation in 1 mM ascorbic acid solution to stabilize to color and the avoid excessive background color. The gels thus treated were placed in deionized water overnight and transferred to 50% methanol for storage. The technique is described in more detail by Benjamin and Montgomery (1973).

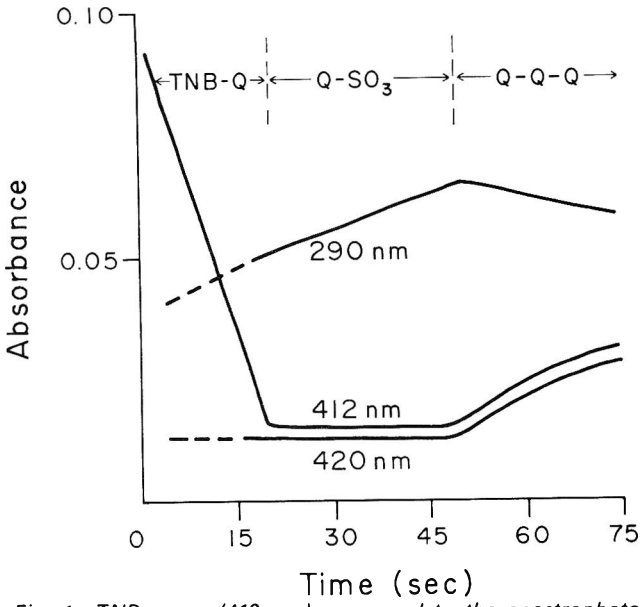

Fig. 1—TNB assay (412 nm) compared to the spectrophotometric method (290nm and 420 nm) for PPO activity in the presence of sulfite. TNB-Q: product formed between TNB and quinones. Q-SO_3: quinone-sulfite derivative. Q-Q-Q: polymerization of quinones. Conditions were 2,500 units of activity/mL of mushroom PPO and either none, or 0.1 mg/mL, of sulfite, pH 5, and 20°C.

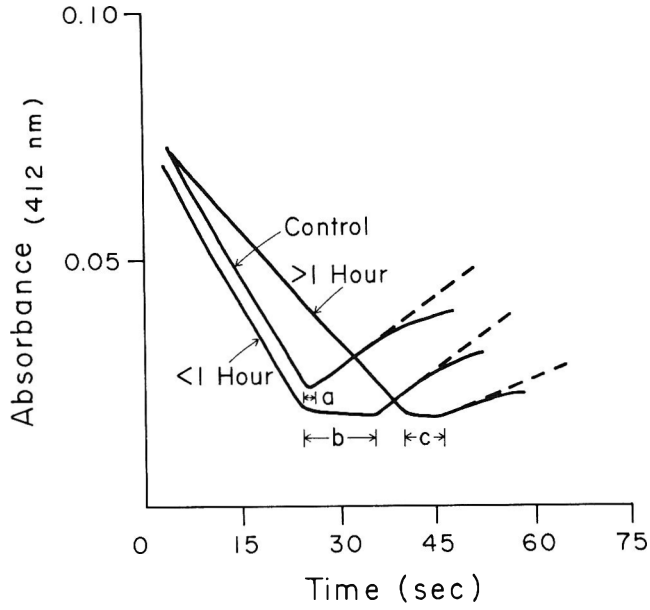

Fig. 2—Activities of PPO as determined by the TNB method. (a) activity of PPO without sulfite at zero time; (b) activity of PPO with sulfite at zero time; and (c) activity of PPO with sulfite after 60 min. Conditions were 2,500 units of activity/mL of mushroom PPO and either none, or 0.1 mg/mL, of sulfite, pH 5, and 20°C. Note absence of lag period without sulfite and decrease of lag period with increased time of incubation.

Stain for protein in polyacrylamide gels

The method used was a variation of that designed by Wray et al. (1981). All solutions were prepared using distilled-deionized water. The gels were fixed after electrophoresis with 10% trichloroacetic acid (TCA) for 30 min, rinsed thoroughly with water to remove excess TCA and soaked overnight in 50% methanol-0.05% formaldehyde. Gels were then placed in water and allowed to swell 30 min with two water changes and transferred again to 50% methanol-0.05% formaldehyde for 2 hr. Immediately before adding the staining solution the gels were placed in deionized water for 10 min. The staining solution was prepared by adding 4g silver nitrate in 20 mL of water to 105 mL of 0.36% sodium hydroxide plus 10 mL of concentrated ammonium hydroxide, then rapidly mixed. The solution thus prepared was made up to 500 mL with deionized water and used within the next 5 min. To assure even staining, the gels were shaken gently for 20 min.

To develop the gel, the staining solution was discarded and the gels rinsed for 5 min in water, followed by a solution of 2.5 mL 1% citric acid, 0.25 mL of 38% acetaldehyde and water to make 500 mL. The slabs were gently agitated until bands of protein were evident. The developing solution was then discarded and the gels rinsed with water. To decrease background intensity, Kodak Rapid Fixer (Eastman Kodak Co.) was prepared and used according to the directions of the manufacturer. Then the destaining step was stopped by rinsing with water and adding Kodak Hypo-clearing agent (Eastman Kodak Co.) prepared as directed by Kodak. The gels were stored in 50% methanol.

Source and analysis on sulfite

Aqueous solutions of sodium sulfite (Sigma) were used. Fresh solutions were prepared prior to each experiment performed. To analyze for sulfite, a modification of the method developed by Wedzicha and Bindra (1980) was employed. An aliquot of the solution containing sulfite was placed in a buffered solution (0.02M phosphate buffer, pH 8.0) containing 5,5′-dithiobis(2-nitrobenzoic acid). The reaction between this reagent and sulfite is quantitative at pH 6–9 to give, as one of the products, 5-mercapto-2-nitrobenzoic acid (extinction coefficient 15,500 at 412 nm when present as a thiol anion). A standard curve was used to determine sulfite in mg/mL in the unknowns. Enzyme solutions were assayed for sulfite in the absence of substrate.

Preparation of labeled SO_2

Labeled sulfur dioxide ($^{35}SO_2$) used in this experiment, was purchased as a gas (Amersham Radiochemicals, IL, product SJ.24), or generated from sulfuric acid labeled in the sulfur atom (New England Nuclear, MA, product NEX-042). The $^{35}SO_2$ was generated by boiling the labeled sulfuric acid in the presence of metallic copper and trapping the gas in 0.02M phosphate buffer, pH 8. The$^{35}SO_2$ generated in the boiling flask was displaced with a flow of nitrogen through the system which passed thorugh two condensers to avoid water or sulfuric acid contamination in the receiving flask, which was kept in crushed ice. Concentration of sulfite was determined and used immediately or stored at 4°C to minimize losses.

Sulfite-enzyme treatments

Enzyme with similar levels of activity (1000–3000 units of activity/mL) were exposed to sulfite (0–0.3 mg/mL). Experiments for the effect of time of exposure to sulfite on the enzyme preparations were performed at pH 5 and incubated at 20°C. Experiments for the effect of pH levels on inactivation of PPO were performed at a constant concentration of sulfite (0.1 mg/mL) and incubated at 20°C. Aliquots of the treated enzyme preparations were assayed for their activity using the standard TNB method.

Radiolabeled SO_2 treatments were performed in a fashion similar to the nonradiolabled treatments, unless otherwise indicated.

Detection of labeled sulfur

Two techniques were used to detect ^{35}S: liquid scintillation counting (LSC) and autoradiography. Samples for LSC were aliquots of the fractions collected during elution of the purification columns (see procedure by Wissemann and Montgomery, 1985). Alternatively, slices of gels were counted directly by swelling the gel slices with Protosol (New England Nuclear, MA) or digesting the gel slices in 60% perchloric acid and 20% hydrogen peroxide (Mahin and Lofberg, 1966) before LSC. Autoradiographs were enhanced by using EN^{3}HANCE (New England Nuclear, MA). The printing was on a blue sensitive X-ray film (Kodak X OMAT AR film) in a wafer rigid form cassette (7×17 Hasley X-ray products) for 7 days at −70°C. The film was developed following the directions of the manufacturer.

Generation of the apoenzyme

Apoenzyme of PPO was prepared using the procedure of Makino et al. (1974) with slight modifications. The enzyme was dialyzed against 0.05M phosphate buffer (pH 7) for 12 hr with one buffer change to remove sulfites if present. Once the sulfites were removed, the enzyme was dialyzed in a Spectrapor membrane (molecular weight cutoff: 10,000–12,000) against 0.05M potassium cyanide in 0.1M phosphate buffer, pH 7, for 10 hr (longer dialysis was found to be

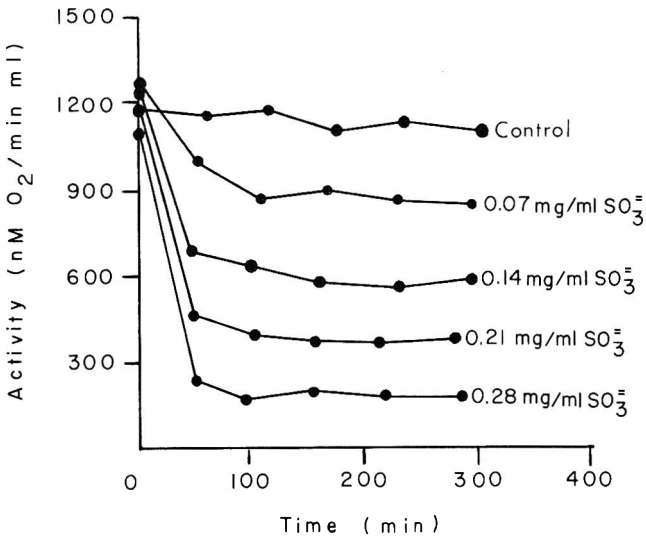

Fig. 3—Effect of the concentration of sulfite on purified pear PPO activity. Conditions were 1,000 units of activity/mL incubated at 20°C in 0.1M citrate-0.2M phosphate pH 5. The TNB method was used to assay for activity.

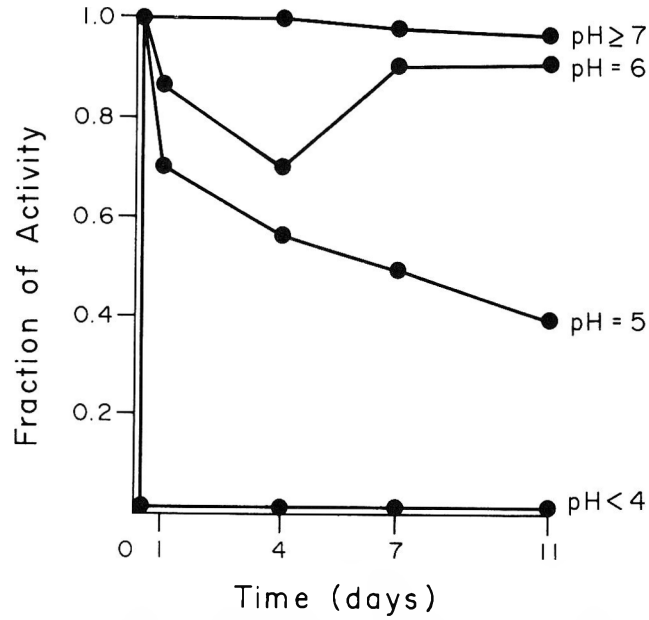

Fig. 4—Effect of pH on purified pear PPO isoenzymes activity at fixed concentration of sulfite. A volume of 5 ml containing approximately 1,500 units of total PPO activity, 0.1 mg/mL of sulfite, and incubated at 20°C was used. PPO activity was determined by the TNB method.

detrimental for regeneration of enzyme activity). Excess potassium cyanide was removed by dialyzing the enzyme 10–12 hr in 0.005M phosphate buffer, pH 7. The enzyme was reactivated by adding cupric sulfate in equimolar concentrations (Kertesz, 1966).

Activities were assayed by the standard TNB method or by following oxygen consumption using the oxygen electrode (Cooper, 1977). Conditions and concentrations of reactants were kept similar to the standard TNB procedure. Units were expressed as nMols of diphenol transformed to *o*-quinones (0.5 moles of oxygen consumed equal one mole of *o*-quinone formed).

RESULTS & DISCUSSION

Assay method

Presence of sulfite in the PPO assay solution complicated the analysis of PPO activity. To compensate for this more than one method of assay was used in the different experiments. The methods tested were: (a) the spectrophotometric method, which measures the formation of polymeric color, (b) the Warburg method and (c) the oxygen electrode, which both measure oxygen consumption, and (d) the method designed by Esterbauer et al. (1977) using 2-nitro-thiobenzoic acid (TNB) as a coupling agent.

The spectrophotometric and the Warburg methods are not sensitive at detecting low enzyme activities. The former also has a period of no change in absorbance in the presence of sulfite (Embs and Markakis, 1965), causing lack of precision. SO_2 reacts with the elements of the oxygen electrode, which may lead to erratic readings (Cooper, 1977).

Because of the disadvantages of other methods over the TNB method, the latter was chosen to perform most of the enzyme assays in this work. The TNB method permits the assay of PPO activity without the development of polymerized brown products. This is because the *o*-quinones react immediately with the TNB reagent and do not participate in further reactions. There is a deviation from linearity toward the end of the reaction, which Esterbauer et al. (1977) suggested was caused by the *o*-diphenols or some intermediates of enzyme oxidation acting as inhibitors of the reaction and not due to the lack of substrates. This observation agrees with other results (Mayer et al., 1966). The spectrophotometric method was used to compare the slopes obtained at 420 nm, with those from the increase in absorption in the TNB procedure, after all the coupling reagent had been consumed at 412 nm. The slopes were found to be the same for similar activities (Fig. 1).

The TNB method gave reproducible results. A deviation from linearity was noticed only when sulfite was present in the reaction vessel at concentrations above 1.0 mg/mL. Assaying the enzyme with this method, three different phenomena could be differentiated (Fig. 1): first, the reaction of the *o*-quinones with the TNB reagent (TNB-Q section) where the rate of decrease in absorbance depended on the PPO activity; secondly, in the section labeled "Q-SO_3" a period of no change in absorbance in assay solutions containing sulfite was observed (Fig. 1). This period corresponds to that observed by Embs and Markakis (1965) and was accompanied by an increase in the absorbance at 290 nm, presumably due to *o*-quinones-sulfite complex formation. Thirdly, Fig. 1 also shows the decrease in absorbance at 290 nm and increase at 412 nm followed by color formation due to the polymerization of quinones and phenols (Embs and Markakis, 1965). Absorbance at 420 nm followed the absorbance at 412 nm in the TNB method (Fig. 1).

After a 60 min period of incubation of the enzyme in the presence of sulfite, the period of no change in absorbance observed at 412 nm in the TNB method or 420 nm in the spectrophotometric method decreased (Fig. 2), probably due to the loss of SO_2 to the atmosphere or to the reaction of sulfite with the enzyme. At this time loss in the PPO activity was also noticed (Fig. 2). The portion of no change in absorbance of this curve was proportional to the concentration of sulfite. At all instances, incubation of the enzyme in the presence of sulfite was performed in the absence of substrate.

Effect of time of exposure to sulfite

To determine the effect of sulfite concentration on PPO with time, similar levels of enzyme activity were exposed to increased levels of sulfite and incubated. Aliquots of these preparations were assayed for activity at various times. Data in Fig. 3 revealed that the levels of inactivation were correlated with the concentration of sulfite present in the enzyme solution and that the inhibition increased with time and reached a plateau within 100 min. Once in this plateau stage, inactivation occurred at a much slower rate. In some instances, as when pH greater than 6 was used in the incubation mixture, more than 24 hr were required to attain the plateau. At pH 5 concentrations above 0.28 mg/ml of sulfite were necessary to com-

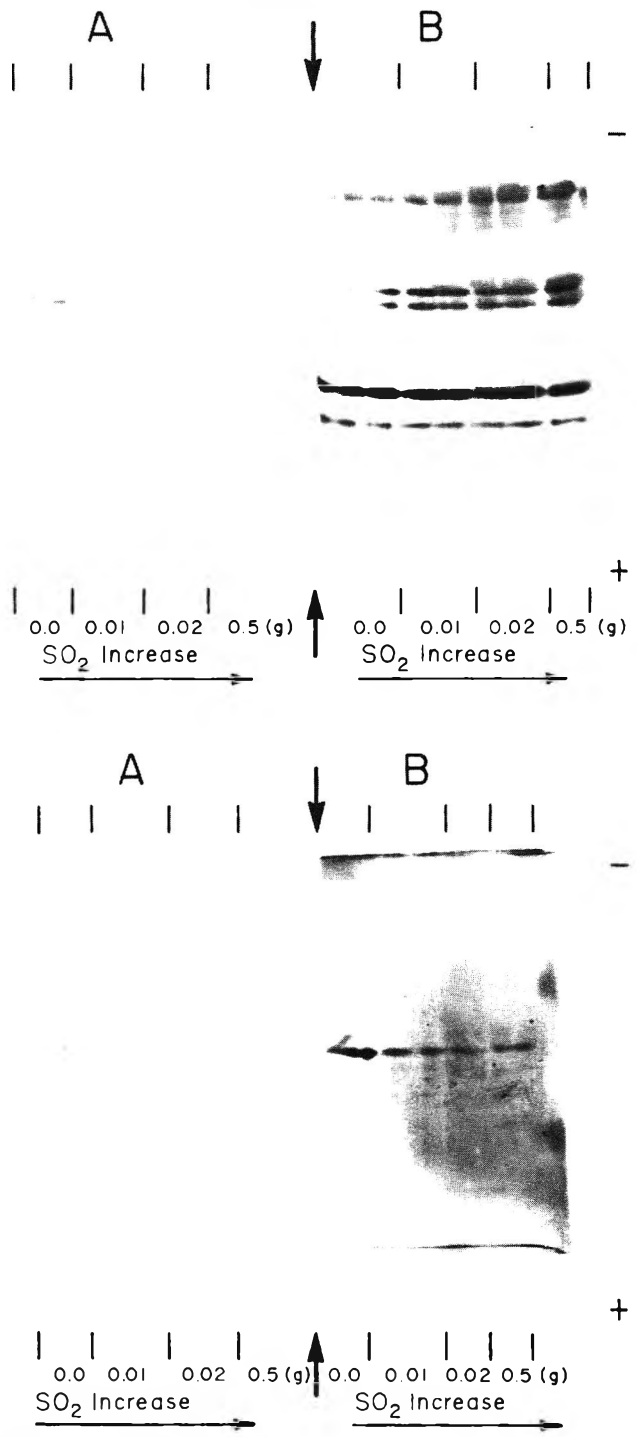

Fig. 5—Polyacrylamide electrophoresis on 7% slab gels of PPO at increased concentrations of sulfite with no incubation time at pH 7. Top: purified pear PPO isoenzymes showing three protein impurities. Bottom: a pure pear PPO isoenzyme. Stains for (A) PPO activity and for (B) protein are shown.

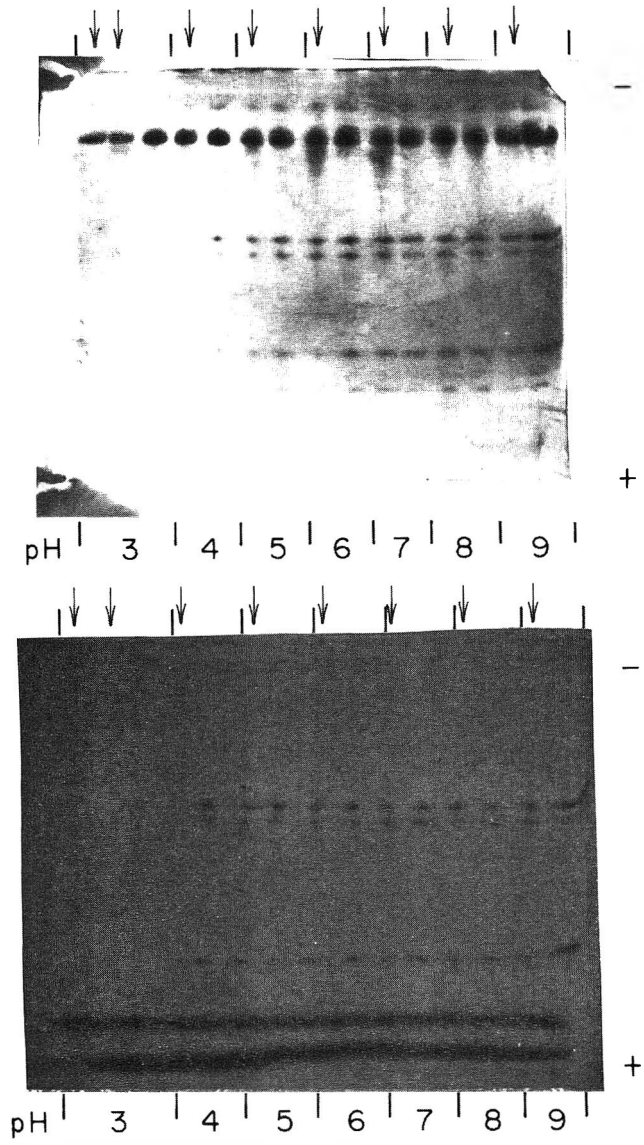

Fig. 6—Polyacrylamide electrophoresis on 7% gel of active pear PPO isoenzymes at constant concentration of sulfite. Incubation time was 30 min at 20°C and different pH levels. Stains for (top) protein and for (bottom) PPO activity are shown. Arrows indicate the sulfite-treated samples. Continuous bands at the bottom of the gel for PPO activity were due to the solvent front. Concentrations were 0.1 mg protein/ml containing 300 units of activity/ml approximately, and 0.04 mg of sodium sulfite/mL.

pletely inactivate the enzyme.

The formation of the plateau and the slow inactivation that occurred once this stage was reached may suggest an equilibrium:

$$[E] + HSO_3^- \rightarrow [E]i\text{-}SO_3 <<<\rightarrow [E]i + HSO_3^-$$

where [E] stands for the initial concentration of active enzyme, $[E]i\text{-}SO_3$ for the possible complex formed between the inactive enzyme and HSO_3^- (see experiments involving electrophoresis), and [E]i for the inactive enzyme. The inhibition of the enzyme, as seen in the experiments of PPO activity regeneration, could be stated as irreversible and that the inactive enzyme with HSO_3^- could be in equilibrium. The stability of $[E]i\text{-}SO_3$, if formed, apparently was low because its existence could not be demonstrated in this work (see experiments involving $^{35}SO_2$).

As observed in this experiment, a time of incubation was required to induce a certain degree of inhibition, which could suggest a low affinity of the enzyme for sulfite. At higher concentrations of sulfite the inhibition was enhanced because of the displacement of the reaction to the right until most of the HSO_3^- had been used. From this stage the inhibition was slower because HSO_3^- probably came only from the dissociation of the complex $[E]i\text{-}SO_3^-$.

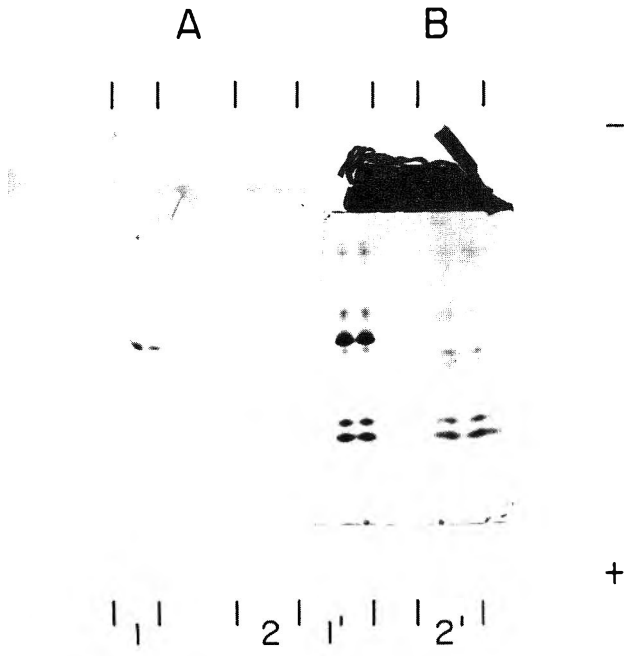

Fig. 7—Polyacrylamide electrophoresis on 7% slab gel of a pear PPO isoenzyme before and after being completely inactivated by 0.3 mg/mL of sulfite, pH 5, for 60 min at 20°C. A: shows the gel stained for PPO activity; B: shows the gel stained for protein. Even numbers are the sulfite treatments.

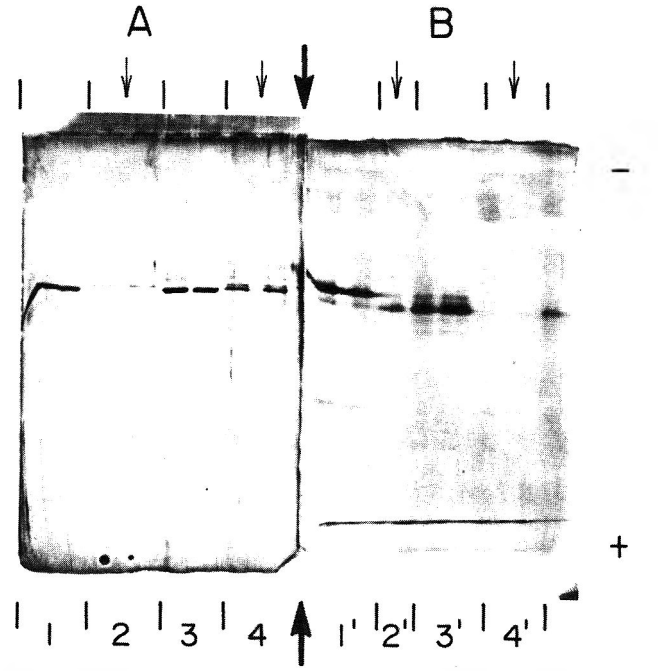

Fig. 8—Polyacrylamide electrophoresis on (A) SDS 10% slab gel and (B) 7% polyacrylamide slab gel of a pure pear isoenzyme before and after being exposed to 0.3 mg/mL of sulfite, pH 5, for 60 min at 20°C. Gels were stained for protein. Even numbers are the sulfite treatments.

Effect on pH on inactivation of PPO

Sulfite did not inhibit PPO at pH levels equal or greater than 7 unless the concentration was increased above 2.0 mg/mL. As the pH of the PPO solution was decreased, inhibition by sulfite increased until at pH levels below 4, inhibition was instantaneous and complete with sulfite concentrations of 0.04 mg/mL (Fig. 4). At pH 5 inhibition of PPO was progressive (after rapid initial loss of activity during the first day of storage) over the 11 days storage period. Enzyme activities were compared to a control containing no sulfite (for all pH values tested) and were reported as a percent of the original activity.

Similar results were obtained with PPO from the three different sources tested (banana, mushroom, and pear). In the sulfite system, the concentration of the HSO_3^- specie increases as the pH is decreased (Green, 1976). This and the results in Fig. 4 would suggest that HSO_3^- was the major component of the sulfite system responsible for the inhibition of PPO at pH 5 and below. Muneta and Wang (1977) observed similar results with a crude extract of potato PPO. An explanation for the apparent inactivation and reactivation of PPO activity exposed to sulfite at pH 6 between day 1 and 7 (Fig. 4) is not readily apparent. Data in the following section show that PPO was not regenerated after inactivation with sulfite.

It is worthwhile to mention that there is a resemblance in the amino acid composition of polyphenoloxidases that had been isolated from a variety of sources (Lerch, 1978; Flung et al., 1963; Jolley et al., 1969). Very low or negligible amounts of sulfur-containing amino acids are reported by these authors. Furthermore, Lerch (1978) reported an unusual thioether linkage between a single cysteinyl residue and a histidyl residue in PPO of *Neurospora crassa*, suggesting that this could be important in the enzyme activity.

In addition, data obtained from the different sulfite inhibited PPO sources used in this work and the electrophoresis of treated pear samples, suggest a similar mode of action for most polyphenol oxidases. The main action of sulfite on the enzyme might be sulfitolysis at a vital point for enzyme activity. The mode of action could be similar to 2-mercaptoethanol (Assa et al., 1978) but due to the instability of the S-sulfoprotein (Cole, 1967), if produced, binding of sulfite to PPO was not possible to detect in this research.

Regeneration of PPO activity after sulfite exposure

Attempts to regenerate PPO activity after inhibition by sulfite were not successful. Dialysis of sulfite inactivated purified pear PPO against 0.01M phosphate (pH 7) for 15 hr, or until no sulfite could be detected, did not restore PPO activity. Column chromatography of purified pear PPO inactivated with sulfite on Phenyl Sepharose or DEAE-cellulose, as performed during the extraction procedure (Wissemann and Montgomery, 1985), did not regenerate the PPO activity. Addition of copper salts to solutions of sulfite-inactivated PPO where the excess sulfite had been removed by dialysis, or to solutions of sulfite-inactivated PPO apoenzyme, did not return the activity.

Thus the inactivation of PPO by sulfite was not reversible (Dixon et al., 1979). Therefore the inhibition pattern cannot be treated by classical kinetics. A PPO-SO_3 complex could have occurred due to the interaction between sulfite and PPO, forming inactive PPO that differed in some properties. This was shown by the additional bands on electrophoresis. Formation of the complex between the *o*-quinones and sulfite, when present in the TNB and spectrophotometric methods, was suggested by the presence of a lag period before the onset of the browning. This has also been demonstrated by Embs and Markakis (1965).

Using purified pear PPO inhibited with $^{35}SO_2$, nearly 100% of the radioactivity could be recovered from the reaction mixture by using the above methods. No labeled SO_2 was detected in the inactivated PPO fractions from column chromatography on Phenyl Sepharose and DEAE-cellulose, as performed in the extraction procedure of Wissemann and Montgomery (1985). Hence, nearly complete removal of sulfite was accomplished by these methods and, in all cases, no regeneration of the PPO activity was observed. Therefore, if $^{35}SO_2$ was bound to the purified pear PPO, it was removed by the above methods or was present in concentrations not detected by the methods used in this work.

Electrophoresis of PPO inactivated by sulfite

Purified pear PPO isoenzymes of known electrophoretic pattern were treated with increasing concentrations of sulfite at constant enzyme activities. Immediately after sulfite exposure and after at least 60 min incubation time, the samples were subjected to electrophoresis on 7% polyacrylamide gel. Results (Fig. 5) showed that at zero time as the concentration of sulfite was increased the staining for PPO activity decreased. Inactivation of PPO by sulfite at pH 7 and after a short time of incubation is minimal or none at all (Fig. 4 and Fig. 6).

The gel stained for protein showed no changes in the intensity of the protein bands in the samples treated with sulfite. Additional protein bands in the samples that were treated with 0.025 and 0.50 mg/mL of sulfite were noticed (Fig. 5). The presence of additional bands could also be observed when purified pear (PPO) isoenzymes treated with sulfite were applied to the gel (Fig. 5). As the enzyme became more inactivated by the presence of sulfite, the formation of additional bands became more evident. Excess sulfite was added to some of the wells to determine if sulfite was interfering with the silver stain. Visually, the color of the stained bands changed, but the intensity of the bands remained approximately the same. Therefore, at the concentrations used in this experiment, sulfite did not appear to interfere with the protein staining procedure.

Figure 6 shows gels stained for protein and PPO activity of an active PPO isoenzyme treated at fixed levels of sulfite and at different pH levels. Activity assays of the PPO-sulfite solutions at pH 5 by the standard TNB method showed that after 30 min at pH lower than 4, complete inactivation of PPO occurred. The samples before being applied to the gels were adjusted to pH 6–7. From Fig. 6 it is evident that the disappearance of the PPO bands in the samples treated with sulfite at the lower pH levels was due to the interaction of sulfite with the enzyme and not to the interference of sulfite during the staining technique. Proteins other than PPO were less affected by sulfites as seen by the lack of change in the protein contaminants at the top of these gels. These same samples were also used to determine the effect of sulfite on PPO activity with pH (Fig. 4).

To gain more evidence for the formation of additional protein bands in PPO preparations treated with sulfites, an isolated pear isoenzyme was used. The activity was completely inhibited with sulfite at pH 5. The sulfite was dialyzed out of the inactivated PPO solution with 5 mM phosphate buffer, pH 7. The sample was subjected to electrophoresis (Fig. 7) and compared with a fully active sample. In the sulfite-treated samples stained for protein and PPO activity, the disappearance of the band where PPO activity should have been present with reference to the control was evident. The gel stained for protein showed additional protein bands which were in some cases also of decreased intensity. This could probably be attributed to the dilution of the protein on the gel as the number of bands increased. Similar gels with $^{35}SO_2$-treated samples did not show radioactivity in these bands after autoradiography.

These samples were also subjected to 10%% SDS electrophoresis. The relative mobility of the samples treated and not treated with sulfite did not show any significant changes in mobility. This observation suggested that the action of sulfite was modifying PPO in such a way that it cannot regain its original form nor activity, while still retaining its molecular weight (Fig. 8).

CONCLUSIONS

FOR THE ASSAY of enzyme activity in the presence of sulfite, the TNB method proved to be the most adequate. Increasing the concentration of sulfite did progressively inhibit the enzyme, and once the activity was stabilized after the initial inhibition (approximately 100 min), inactivation occurred at slower rates. Lower pH reduced the concentration of sulfite needed to completely inactivate the enzyme. Trials to reactivate PPO after inhibition with sulfite were unsuccessful giving evidence of an irreversible inhibition of PPO by sulfite.

Polyacrylamide gels gave evidence of the modification of the structure, and/or change of ionization, of the enzyme once it was inactivated by sulfite. Two additional protein bands and the disappearance of the protein band where the active PPO should have been were noticed. SDS gel electrophoresis showed that the molecular weight of the enzyme remained the same or was only slightly modified before and after treatment with sulfite.

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Effective Molecular Weight of Aqueous Solutions and Liquid Foods Calculated From the Freezing Point Depression

C. S. CHEN

ABSTRACT

Freezing point depression (FPD) methods were applied to estimate the effective molecular weights of pure solutions and liquid foods. Explicit expressions among FPD, molecular weight and concentration were developed. The estimated molecular weights were about 333–356 for skim milk, 347–350 for coffee beverage, 164–167 for grape juice and 196–203 for tomato juice. The FPD methods were considered accurate for nonacidic foods but they could underestimate the values for acidic foods.

INTRODUCTION

MANY LIQUID FOOD PRODUCTS are processed into concentrated or dehydrated forms. Some familiar concentrated foods are milk (24–36% solids), tomato paste (30–32% solids) and orange juice (40–65% solids). Some dehydrated foods are made from liquid extracts such as coffee and tea.

Liquid foods can be considered as complex aqueous solutions. The study of aqueous solutions has been based on the thermodynamic equations of ideal solutions. Real solutions approach the behavior of ideal solutions only at dilute concentration. To account for the deviation from ideality, the common practice is to introduce an activity coefficient. No information on activity coefficient has been reported for liquid food systems. Lack of knowledge on the molecular weights of food systems may have limited the theoretical study on the physical properties of foods, and on how the behavior of foods deviates from the ideal solution laws.

Foods do not freeze at a constant temperature, instead they freeze in a zone of temperatures below O°C. The behavior of freezing point depression (FPD) indicates the properties of solutes in the solution. Bartlett (1944) has applied the ideal FPD equation to characterize the food systems using the initial freezing point (the highest freezing temperature of the freezing zone). Schwartzberg (1976) has developed a modified equation and a method for characterizing the foods using multiple sets of freezing data. The possibility of applying the technique of the FPD for systematic determinations of molecular weight for aqueous solutions and liquid foods was investigated.

The objectives of this study were: (1) to investigate the applicability and limitations of the FPD methods for molecular weight determinations, and (2) to develop relationships of FPD, molecular weight, and concentration for real solutions.

THEORY

Model 1—the ideal solution laws

For the ideal dilute solution, Raoult's law of water activity is given as:

$$a_w = \frac{1-X_s}{1-X_s + EX_s} \quad (1)$$

Author Chen is with the Institute of Food & Agricultural Sciences, Citrus Research and Education Center, Univ. of Florida, 700 Experiment Station Road, Lake Alfred, FL 38850.

Table 1—Curve fitting of freezing point depression data for some solutions of pure substances[a]

Substance	Coefficents of Eq. (11)			Range of Index	
	C_1	C_2	C_3	X_s	r^2
Citric acid	10.290	9.475	12.749	0-0.30	0.993
Ethanol	31.808	157.439	−141.850	0-0.68	0.982
Formic acid	42.750	−5.749	66.584	0-0.64	0.990
D-fructose	10.282	10.693	22.902	0-0.40	0.998
D-glucose	10.547	7.991	34.200	0-0.30	0.999
Glycerol	18.702	38.635	26.429	0-0.40	0.993
Inulin	0.528	−2.341	30.605	0-0.10	0.968
Lactic acid	18.262	19.129	8.384	0-0.20	0.979
Lactose	5.639	−4.588	140.566	0-0.08	0.995
Maltose	5.661	1.443	29.939	0-0.44	0.998
Methanol	57.917	65.289	79.799	0-0.68	0.997
Sucrose	5.629	2.743	27.415	0-0.42	0.998
Urea	29.540	31.484	−14.803	0-0.44	0.990
Sodium chloride	59.593	2.473	559.546	0-0.23	0.999

[a] Data source: Weast (1985).

where a_w is water activity; X_s is solids (kg/kg solution); E is the ratio of the molecular weight of water (M_w) to the molecular weight of solutes (M_s).

Based on the method of Bartlett (1944), the following equation has been derived (Chen, 1985a,b).

$$EX_{so} = X_{wo}\,(e^{-a_1 t_1} - 1) \quad (2)$$

where X_{so} is initial solids (kg/kg solution); X_{wo} is initial moisture (kg/kg solution); $a_1 = L_o M_w / RT_o^2$, in which L_o is latent heat of fusion of water at 0°C, R is ideal gas constant (= 1.987 Kcal/kg-mole °K), T_o is freezing point of water (= 273.1 °K); $t_1 = T_1 - T_o = -\Delta t_o$, in which t_1 is initial freezing temperature in °C, T_1 is initial freezing temperature in °K and Δt_o is the initial FPD.

It should be noted that the temperature coefficient of enthalpy of melting has been neglected in the expression of Eq. (2). For more general expression of freezing point depression, several excellent books on chemical thermodynamics should be consulted (e.g., Lewis and Randall, 1961; Denbigh, 1966).

Using $e^{-a_1 t_1} \approx 1 - a_1 t_1$, from Eq. (2), it can be shown:

$$EX_{so} = X_{wo}\, a_1\, \Delta t_o \quad (3)$$

Recall $E = M_w/M_s$, from Eq. (3), the following explicit equation for M_s can be derived:

$$M_s = \frac{(RT_o^2/L_o)\, X_{so}}{X_{wo} \cdot \Delta t_o} \quad (4)$$

Denoting $K = RT_o^2/L_o$ and using $X_{wo} = 1 - X_{so}$, Eq. (4) becomes:

$$M_s = \frac{KX_{so}}{(1-X_{so}) \cdot \Delta t_o} \quad (5)$$

The value of M_s can be readily determined from the initial values of X_{so} and Δt_o. It should be noted that $K = 1860 = 1000\, K_f$ in which K_f is the FPD constant of water (K_f = 1.86 Kg°K/kg mole). The values of K_f for various solvents are widely available (Dainels et al., 1970).

Eq. (5) can be expressed in a general form as:

$$M_s = \frac{KX_s}{(1-X_s) \cdot \Delta t} \quad (6)$$

Eq. (6) can be used to evaluate M_s from any set of freezing point data (ideal solution assumed).

Table 2—A comparison of results of pure solutions calculated from various equations

Substance	Formula mol. wt M_s (1)[a]	Model 1 M_s (2)	Model 1 % dev. (3)	Model 1 M_s (4)	Model 1 % dev. (5)	Model 2 b (6)	Model 2 M_s (7)	Model 2 % dev. (8)	Model 2 b (9)	Model 2 M_s (10)	Model 2 % dev. (11)
Citric acid	192.12	181.29	−5.6	183.42	−4.5	−0.07	180.12	−6.3	0.30	198.19	+3.2
Ethanol	46.01	49.79	+8.1	42.06	−8.7	0.10	43.74	−5.2	0.29	47.16	+2.4
Formic acid	46.03	45.93	−0.2	62.27	+36.2	−0.37	47.83	+3.9	−0.47	45.17	−1.9
D-fructose	180.16	180.04	−0.1	175.41	−2.6	0.14	181.36	+0.6	0.10	179.59	−0.3
D-glucose	180.16	177.47	−1.5	172.12	−4.5	0.18	180.11	−0.2	0.18	180.16	0
Glycerol	92.09	94.58	+2.7	83.30	−8.5	0.22	91.61	−2.2	0.23	92.22	+0.1
Inulin	5200.00	4014.23	−22.8	3787.30	−27.2	0.75	3988.88	−23.3	5.19	6222.06	+19.7
Lactic acid	90.08	101.76	+12.9	102.52	+13.8	−0.09	101.12	+12.3	−1.26	86.91	−3.5
Lactose	342.30	339.84	−0.7	337.17	−1.6	0.47	345.82	+1.0	0.21	341.02	−0.4
Maltose	342.29	337.09	−1.5	307.29	−10.2	0.28	349.09	−2.0	0.21	337.08	−1.5
Methanol	32.03	31.90	−0.4	34.65	+9.9	−0.15	30.08	−6.1	−0.02	33.97	+6.1
Sucrose	342.30	335.55	−2.0	305.61	−10.7	0.28	344.56	+1.0	0.26	340.33	−0.6
Urea	60.06	63.00	+4.9	71.19	+18.5	−0.45	59.26	−2.5	−0.44	60.04	0.0
Sodium chloride	58.44	32.04	−45.2	29.88	−48.9	0.62	33.85	−42.1	−0.47	27.74	−52.5
RMS[b]			3.4		10.8			3.6			1.7

[a] Columns: (1) from Weast (1985); (2) and (3) evaluated from Eq. (5) and 5% concentratin; (4) and (5) evaluated from Eq. (6) for the observed range; (6), (7), and (8) evaluated from Eq. (8) and (9); (9), (10), and (11) evaluated from Eq. (8) and (10).

[b] RMS = Root mean square error excluding inulin and sodium chloride.

Table 3—A comparison of experimental and calculated FPD data for sucrose solution

Conc (% by wt) X_s	Expt data[a] Δt (°C)	Calculated values Δt^b (°C)	Calculated values Δt^c (°C)
0.50	0.027	0.028	0.027
1.00	0.055	0.057	0.055
10.00	0.625	0.618	0.623
20.00	1.465	1.455	1.461
30.00	2.644	2.676	2.646
40.00	4.452	4.445	4.454
42.00	4.932	4.879	4.936

[a] Data from Weast (1985).

[b] Δt calculated from Eq. (11) using $C_1 = 5.629$, $C_2 = 2.743$, $C_3 = 27.415$.

[c] Δt calculated from Eq. (12) using $b = 0.28$.

Model 2—absorption and liberation of free water

Based on the concept of bound water, Schwartzberg (1976) modified Eq. (1) to the following expression:

$$a_w = \frac{1-X_s-bX_s}{1-X_s-bX_s+EX_s} \tag{7}$$

where b is the amount of water which is bound per unit weight of solids and is unavailable for freezing at any temperature. We assume that the water activity can also be increased due to complex interaction between solutes and water and it can be expressed by the equivalent increase in free water. Thus, the value of b can either be positive or negative depending on the behavior of freezing point data.

Replacing the term $(1-X_s)$ in Eq. (6) by the term $(1-X_s-bX_s)$, the following expression is obtained:

$$M_s = \frac{KX_s}{(1-X_s-bX_s)\cdot\Delta t} \tag{8}$$

Eq. (8) is the corresponding modified FPD equation.

Determination of b and M_s

Eq. (8) contains two unknown variables: b and M_s. They can be determined from two sets of data X_s and Δt, which need to be well-separated, e.g., 10% and 30% concentrations, respectively. Denoting set one as X_{s1} and Δt_1, set two as X_{s2} and Δt_2, the value of b can be calculated from the following expression:

$$b = \left[\frac{X_{s1}\,\Delta t_2 - X_{s2}\,\Delta t_1}{X_{s1}\,X_{s2}\,(\Delta t_2 - \Delta t_1)} - 1\right] \tag{9}$$

The average value of b obtained from the experimental data can then be used to calculate the value of M_s from Eq. (8) or to characterize the effect of dissociation.

For the pure substances, their molecular weights are well known. The value of b can be calculated from the following expression:

$$b = \frac{1}{X_{s1}}\left[1 - X_{s1} - \frac{K\,X_{s1}}{M_s\,\Delta t_1}\right] \tag{10}$$

Curve fitting of data

To minimize the variation of experimental errors, the following polynomial equation was used to fit the FPD data as a function of solids content:

$$\Delta t = C_1X_s + C_2X_s^2 + C_3X_s^3 \tag{11}$$

where C_1, C_2, C_3 are empirical constants.

RESULT & DISCUSSION

FREEZING POINT DEPRESSION data for aqueous solutions of solutes which are of interest in the food area were selected for the analysis. They represent a wide range of molecular weights (32–5200) and include weak acids, bases, various sugars, glycerol and alcohol solutions.

The results of curve fitting of the data are presented in Table 1. The assumed third degree polynomial was adequate to represent the data as indicated by the correlation coefficient (r^2). These empirical fitting equations are applicable in the concentration range specified for each material. These equations were used to generate the data sets for equal spacings for the deter-

Table 4—Curve fitting of freezing point depression data for some liquid foods[a]

Liquid foods	Coefficients of Eq. (11) C_1	C_2	C_3	Range of X_s	Index r^2
Freeze-dried skim milk	5.604	5.125	14.583	0-0.40	0.849
Freeze-dried coffee beverage	6.079	−3.446	28.555	0-0.40	0.999
Concentrated grape juice	11.056	13.333	0	0-0.30	0.797
Dried tomato juice	9.50	3.33	0	0-0.20	0.098
Avg fruit and vegetable juices[b]	10.0	0	50.0	0-0.50	—

[a] Data source: Lerici et al. (1983).

[b] Riedel (1951).

Table 5—A comparison of effective molecular weights of liquid foods calculated from various equations

Liquid foods	Model 1		Model 2	
	M_s (1)[a]	M_s (2)	b (3)	M_s (4)
Freeze-dried skim milk	356.25	319.44	0.11	333.10
Freeze-dried coffee beverage	349.90	339.85	0.01	346.97
Concentrated grape juice	167.03	169.70	−0.16	163.89
Dried tomato juice	202.54	215.06	−0.67	195.52
Avg fruit and vegetable juice	193.37	181.55	0.20	201.98

[a] Columns: (1) evaluated from Eq. (5) at 5% concentration; (2) evaluated from Eq. (6) for the observed range; (3) and (4) evaluated from Eq. (8) and (9), respectively.

mination of b from Eq. (9) and for the determination of M_s from Eq. (6) and (8), respectively.

Table 2 presents a comparison of results of pure solutions calculated from various equations. The calculated values of M_s from Eq. (6) varied only slightly for solids in the range 1–10%. Thus, the values of M_s evaluated at 5% solids and the observed data range are shown for Model 1, respectively. The values of b were calculated from Eqs (9) and (10), respectively. The averaged values of b were then used to calculate the values of M_s for Model 2. The calculated values of b varied somewhat for the same substance indicating the limitations of the FPD method for obtaining a unique value of b. However, the b value in column 9 should be used to characterize the substance if the molecular weight is known. In general, the b values were positive for sugar solutions indicating the absorption of water by these substances. The b values were negative for acids and bases indicating the dissociation of ions. For sugar solutions, the calculated values of M_s agreed with the formula values with remarkable accuracy for both Models 1 and 2. Model 1 was accurate at low concentration but Model 2 was accurate for a wide range of concentrations. Thus, Model 2 can be used to predict the FPD as a function of concentration using the values of b and M_s in Table 2.

The modified FPD equation can be expressed as:

$$\Delta t = \frac{-K}{M_w} \ln \left[\frac{1-X_s-bX_s}{1-X_s-bX_s+EX_s} \right] \approx \frac{KX_s}{(1-X_s-bX_s)\,M_s} \qquad (12)$$

The approximation expression (right-hand) side of Eq. (12) can be used for concentrations in the range of 0-40% for most sugar containing solutions and the logarithmic expression can be extrapolated to higher concentration.

Eq. (12) contains only one parameter b which can be determined from one data set if molecular weight of the solute is known or two data sets if molecular weight is not known. As a comparison, the empirical Eq. (11) consists of three parameters which requires multiple data sets for curve fitting by a statistical method. Thus, Eq. (12) enables us to simplify the experimental requirements for characterizing the freezing point depression behavior.

Table 3 presents a comparison of experimental and calculated FPD data for sucrose solution. The good agreements between the experimental and calculated data from Eq. (12) demonstrated the advantage of Eq. (12) over the conventional curve fitting methods. Similar results were found for other substances using the values of b from column 6 in Table 2. It should be noted that Eq. (12) can also be used to predict the initial freezing point, t, for any concentration. For example, the initial freezing point of sugar solution at 10% concentration is −0.625°C since $t = -\Delta t$. Therefore, Eq. (12) can be utilized as a one parameter FPD and initial freezing point correlation equation.

It is well-known that the ideal Eq. (1) and consequently Eq. (7) are not applicable to electrolyte solutions which have to be treated differently (see Harned and Owen, 1958; Robinson and Stokes, 1959). As can expected, the FPD were not as accurate for weak acids and bases as for sugar solutions. They were not applicable for sodium chloride (an electrolyte solution) and inulin which has small freezing point depression due to large molecular weight. The values of M_s calculated from the FPD methods might differ from the formula M_s with a factor. For example, the factor for sodium chloride is about two which is, known as Van't Hoff coefficient, due to the ionization of sodium and chloride. It is observed that the factor for citric acid is about (1 + b), and lactic acid is about (1-b), in which the values of b are from column 6 in Table 2. These empirical factors need to be further studied since the measurement of the freezing point could also be used as a measure of the degree of dissociation if the molecular weight is known. The measurement errors could also contribute to the variations.

Most liquid foods are mixtures of complex composition which includes various sugars, acids, proteins, fats and minerals. The FPD reflects the overall effect of the various solutes in the solution. Thus, the molecular weight evaluated from the FPD methods is not necessarily the true value but it can represent the behavior of the products as though it has that equivalent molecular weight. The molecular weight determined from the FPD methods is called effective (or apparent) molecular weight.

Table 4 presents curve fitting of FPD data for some liquid foods. The polynomial equations were less accurate for liquid foods compared to pure substance.

Table 5 presents a comparison of effective molecular-weights of liquid foods calculated from various equations. The values of M_s in columns (1) and (4) agree well with only 1–6% differences. The estimated molecular weight for freeze-dried skim milk was about 333–356 which is close to the molecular weight of lactose (molecular weight = 342.30). The estimated molecular weight for grape juice is about 164–167 which was less than the molecular weight of glucose or fructose (molecular weight = 180.16) or sucrose (molecular weight = 342.30). On the basis of the properties of pure substances, it could be inferred that the estimated values of M_s were more accurate for non-acidic foods and less accurate for acidic foods. It is possible that the molecular weights of acidic foods estimated from the FPD methods differ from the true values by a factor which could not be determined from Eq. (9).

Regardless of whether the estimated values of M_s are true molecular weights, the values of b and M_s can be used in Eq. (12) as a semi-empirical FPD equation for correlating the FPD data. There are three advantages for using Eq. (12) over the conventional curve fitting methods. First, the theoretically based Eq. (12) needs only two data sets. Secondly, it can be used to extrapolate beyond the range with more confidence. Thirdly, it can be related to other thermodynamic equations.

CONCLUSION

The principles of FPD can be applied to the study of physical properties of aqueous solutions as well as liquid foods. The ideal FPD equation can be applied to estimate the effective molecular weights of real solutions using the initial freezing point method of Bartlett. The modified equation of Schwartzberg can be extended to higher concentrations. The FPD methods are simple and accurate for molecular weight determinations of non-acidic foods but are not as accurate for acidic foods.

Regardless of whether the effective molecular weights are close to formula molecular weights, the values of b and M_s can be used in Eq. (12) to correlate the FPD data and can be used more advantageously over the conventional curve fitting methods.

—Continued on page 1553

Thermal Transitions in Myosin-ANS Fluorescence and Gel Rigidity

L. WICKER, T. C. LANIER, D. D. HAMANN, and T. AKAHANE

ABSTRACT

Thermal transitions (Tr) in myosin were monitored during constant rate heating with a thermal scanning rigidity monitor (TSRM) and a fluorescent probe, 1-anilino-naphthalene-8-sulfonate (ANS). The Tr values from fluorescent probe measurements were 37°C, 44°C, and 44°C for tilapia, rabbit, and chicken myosin-ANS, respectively. Three Tr values at 43°, 49°C, and 55°C were observed in TSRM measurements of tilapia myosin gelation, whereas a single Tr was observed in rabbit and chicken gelation at 48°C and 49°C, respecitvely. In tilapia myosin, KCl concentration and pH significantly influenced the TSRM but not the fluorescence thermograms. These results indicated that a prerequisite change occurred in the hydrophobic character of myosin just prior to the onset of gelation.

INTRODUCTION

MYOSIN is considered to be the protein primarily responsible for gel formation in muscle foods. At concentrations greater than 3 mg/mL, myosin can form a heat-induced gel and can thus be used as a model system to study the gelling phenomenon (Siegel and Schmidt, 1979a, b). Studies conducted at lower concentrations of myosin can yield information at the molecular level on heat-induced changes in myosin conformation.

The myosin molecule is an asymmetric protein composed of a coiled-coil α-helical tail region and a globular head region. Myosin is considered to be relatively hydrophilic such that electrostatic interactions are thought to be dominant in filament formation based on the distribution of charged amino acids (McLachlan and Karn, 1983) and on the gain of entropy and loss of about 400 mL water/mol myosin in the association process (Josephs and Harrington, 1968). However, myosin has also been shown to have significant hydrophobic character by partition chromatography (Pinaev et al., 1982), by cis-parinaric acid fluorescence (Voutsinas et al., 1983), and by model building from the amino acid sequence (McLachlan and Karn, 1983).

It has been suggested that ANS (1-anilino-naphthalene-8-sulfonate) is an effective fluorescent probe for nonpolar regions of proteins (Stryer, 1965). ANS has been used to detect changes in myosin conformation involving nonpolar residues caused by modification with p-chloromercuribenzoate (Duke et al., 1966), heat (Lim and Botts, 1967), urea, and alkaline pH (Cheung, 1969). Fluorescent probes, such an ANS and cis-parinaric acid, have been successfully used to correlate changes in fluorescence intensity (FI) with functional properties of muscle foods such as ease of low temperature gelation setting (Niwa, 1975; Niwa et al., 1981a,b) and emulsifying capacity (Voutsinas et al., 1983; Li-Chan et al., 1984). In each of these cases it was proposed that an increase in protein surface hydrophobicity as evidenced by an increase in FI was responsible for the measured increase in functional properties.

A primary objective of the present research was to compare changes in myosin conformation detected by ANS fluorescence to those changes which occur in the rigidity thermograms of concentrated solutions of myosin during thermal denaturation for fish and other species. Since the hydrophilic character of myosin would suggest that electrostatic interactions are also important in the denaturation and formation of myosin gels, effects of pH and KCl concentration were also studied with respect to the fluorescence of fish myosin-ANS and rigidity development.

The authors are with the Dept. of Food Science, North Carolina State Univ., Box 7624, Raleigh, NC 27695-7624.

MATERIALS & METHODS

Myosin preparation

Myosin was prepared from pre-rigor tilapia (*Serotherodon aureus*) by a modified method of Akahane (1982). Experiments were completed in less than 1 wk to minimize variation due to aging. Tilapia were obtained from AB Limited, Raleigh, NC. Studies of the pH and KCl effects were conducted on myosin prepared from October to the following May. Studies of species differences utilized myosin prepared subsequently from May to September.

Tilapia fillets were minced and washed several times in a five-fold volume of 50 mM potassium phosphate, pH 6.5. The residue was extracted 15 min at 4°C in 0.3M KCl, 50 mM potassium phosphate, 10 mM $MgCl_2$, 0.5 mM EGTA, and 5 mM ATP, pH 6.5. The slurry was centrifuged at 8,000 × *g* for 20 min, the supernatant was made 3 mM in ATP, and was ultracentrifuged at 110,000 × *g* for 2 hr. The supernatant was diluted with a tenfold volume of distilled, deionized water or dialyzed. The myosin filaments were collected by centrifugation at 8,000 × *g* for 20 min and resuspended in 1.2M KCl, 50 mM potassium phosphate, pH 6.5, and dialyzed to 0.3M KCl, 50 mM potassium phosphate, pH 6.5 before ultracentrifugation at 100,000 × *g* for 45 min. The supernatant was removed and dialyzed against 0.6M KCl, 50 mM potassium phosphate, pH 6.5. Myosin purity and absence of proteolytic digests were evaluated by sodium deodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Porzio and Pearson, 1977). The concentration of myosin was determined by the Biuret method or by absorbance at 280 nm ($\epsilon = 5.5^{\%}_{cm}$, Swenson and Ritchie, 1980). Fish myosin samples were adjusted to the respective pH or KCl concentration by overnight dialysis.

Single comb White leghorns were obtained from the N. C. State University Poultry Science Department. The breast muscle was removed, and myosin was prepared according to the modified method of Akahane (1982) except that precipitation with 10 volumes of distilled, deionized water preceded ultracentrifugation. A New Zealand rabbit was used to prepare myosin from the back and hind leg muscles according to the method of Margossian and Lowey (1982).

Shear modulus

The gelation of myosin was monitored with two similar thermal scanning rigidity monitors (TSRM), which consisted of a water-jacketed cylindrical cup and a serrated, cylindrical spindle (Wu et al., 1984). The first TSRM cup held approximately 10 ml sample and had an inner diameter of 1.90 cm. The spindle diameter and length were 1.548 cm and 4.983 cm, respectively. The second TSRM cup held approximately 5 mL sample and had an inner diameter of 1.250 cm. The diameter and length of the second TSRM spindle were 1.016 cm and 4.617 cm, respectively. In each case, the spindle was attached to a 500g tension load cell mounted in the crosshead of an Instron Universal Testing Machine. Spindles were serrated to avoid slippage in the sample and completely submerged so that buoyancy effects were constant throughout the measurements. Vegetable oil was layered over the suspension to prevent skin formation. Cyclic measurements were taken at a constant displacement of 0.2 cm with a crosshead speed of 0.05 cm/min. Rigidity was calculated as the shear force divided by the shear strain as described by Wu et al. (1984). Transition temperatures were calculated from averages of triplicates from maxima or minima of first derivative plots (dG/dT vs T).

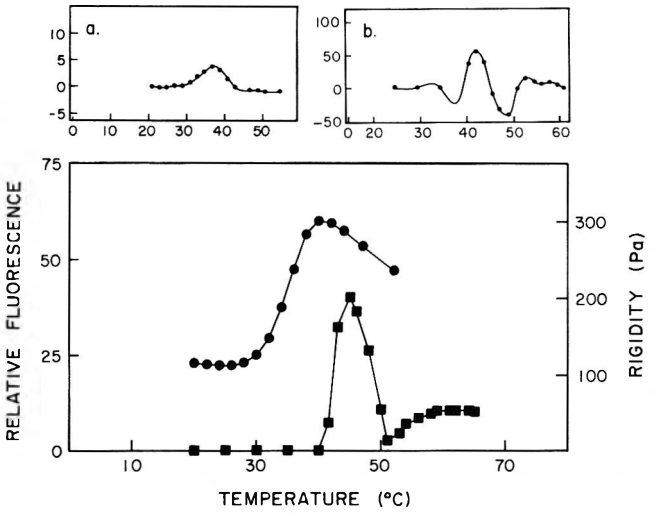

Fig. 1—Fluorescence intensity (●) of tilapia myosin-ANS at 0.5 mg/mL myosin and 32 × 10⁻⁶M ANS. Rigidity of tilapia myosin (■) at 6.4 mg/mL. Inset: First derivative plots of fluorescence (a) and TSRM (b) thermograms.

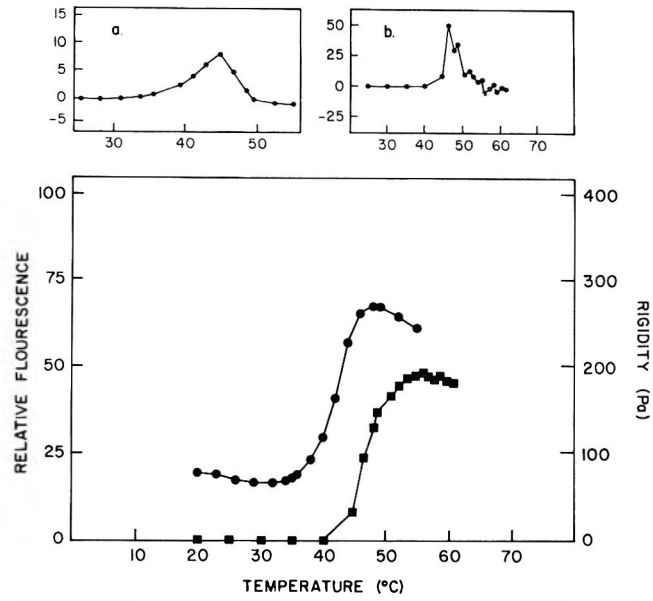

Fig. 2—Fluorescence intensity (●) of rabbit myosin-ANS at 0.5 mg/mL myosin and 32 × 10⁻⁶M ANS. Rigidity of rabbit myosin (■) at 5.3 mg/mL. Inset: First derivative plots of fluorescence (a) and TSRM (b) thermograms.

Fluorescence

Thermal scans of myosin were conducted at a final ANS concentration of 32 × 10^{-6} M and myosin concentrations ranging from 0.025 to 1.0 mg/mL. Cuvettes prepared from capillary tubing, which had an inner diameter of 0.2 cm, were used to minimize light scattering. The wavelengths of excitation and emission were 380 nm and 475 nm, respectively; the excitation and emission slits were 0.5 nm and 16.0 nm, respectively; a 10 sec integration time was used to collect the counts; fluorescence intensity measurements were begun 10 min after ANS addition. Transition temperature was calculated from averages of triplicates from maxima of first derivative plots (dFI/dt vs T).

Temperature control

Water from a Neslab Programmable water bath was circulated around the water jacketed myosin solutions. The heating rate was maintained constant at 1°C/min for both fluorescence and rigidity studies by use of the same bath. The rigidity and fluorescence intensity (FI) values reported at each temperature are the values obtained upon reaching the indicated temperature during the heating cycle. The temperature of the fluorescence sample chamber was monitored directly with a temperature probe. The temperature of the TSRM device was corrected for daily fluctuations in the temperature differential between the water bath and sample chamber.

RESULTS

Rigidity and FI of fish myosin

A typical fluorescence thermogram of tilapia myosin-ANS at 0.6M KCl and pH 6.5 during constant rate heating is plotted in Fig. 1. The FI decreased slightly from 20°C to 27°C, whereupon the FI increased dramatically until 41°C, after which the FI decreased with further increase in temperature. A first derivative plot (dFI/dT vs T) of this data (inset a , Fig. 1) yielded a transition temperature (Tr) at 37°C. Transitions were assigned to peaks which were the result of reproducible shifts in the thermogram.

A typical rigidity thermogram obtained during constant rate heating of tilapia myosin is also plotted in Fig. 1. No change in rigidity was observed between 20–40°C. The rigidity increased sharply in the 41–45°C temperature range, then decreased to 51°C whereupon the rigidity began to increase again before reaching a plateau. Inset (b) (Fig. 1) is a first derivative plot (dG/dT vs T) of this rigidity data which indicates three transition temperatures (Tr). Average values for these transitions were 43°C, 49°C, and 55°C. The first Tr at 43°C was a large, positive transition, whereas the second Tr at 49°C was a large, negative transition and the last Tr at 55°C was a small, positive transition.

Rigidity and FI of rabbit myosin

A typical fluorescence thermogram of rabbit myosin-ANS complex is shown in Fig. 2. Rabbit myosin-ANS underwent a similar increase in FI with increasing temperature as did tilapia myosin-ANS. However, FI did not begin to increase until 32°C and extended to 48°C. A first derivative plot of the data yielded a Tr at 44°C (inset a, Fig. 2). A typical rigidity thermogram of rabbit myosin is also shown in Fig. 2. The rigidity began to increase at 45°C, near the temperature at which the FI of rabbit myosin-ANS began to decrease. A first derivative plot of the TSRM thermograms yielded a single Tr at 48°C (inset b, Fig. 2).

Rigidity and FI of chicken myosin

Typical thermograms of chicken myosin-ANS fluorescence and rigidity of myosin are plotted in Fig. 3. The FI of chicken myosin-ANS increased at 33°C and decreased at 49°C, with a Tr of 44°C (inset a, Fig. 3). The rigidity of chicken myosin increased in a manner similar to that of the rigidity of rabbit myosin beginning at 47°C with a Tr of 49°C (inset b, Fig. 3).

pH effects on FI thermograms of fish myosin-ANS

Fluorescence thermograms of tilapia myosin-ANS at varying pH values are plotted in Fig. 4. At pH values greater than or equal to 6.5, FI increased with temperature with a Tr of 38°C. At pH 6.0, the Tr was 36°C, although the characteristic increase in FI was diminished and the initial FI was higher than the initial FI of myosin-ANS at higher pH values.

pH effects on TSRM thermograms of fish myosin

The effect of pH on the rigidity thermograms of tilapia myosin is shown in Fig. 5. At pH 5.0, myosin was aggregated and the higher initial rigidity reflected this coagulum state. The final rigidity at pH 6.0 was four to five fold higher than that at pH 6.5. At pH 6.5, the initial thermal transitions were more pronounced than at pH 6.0. Myosin formed a very weak gel at pH 8.0 and formed an even weaker gel at pH 7.0 (data not shown). The temperatures at which tilapia myosin began to

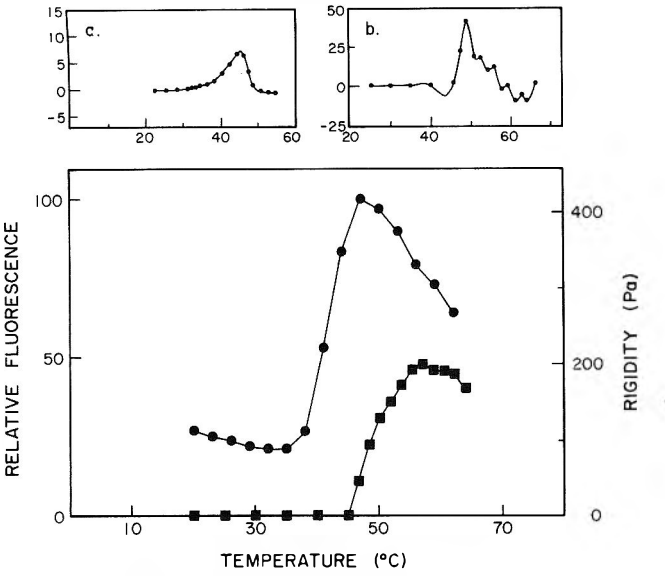

Fig. 3—Fluorescence intensity (●) of chicken myosin-ANS at 0.5 mgiml myosin and 32 × 10⁻⁶M ANS. Rigidity (■) of chicken myosin at 5.9 mg/ml. Inset: First derivative plots of fluorescence (a) and TSRM (b) thermograms.

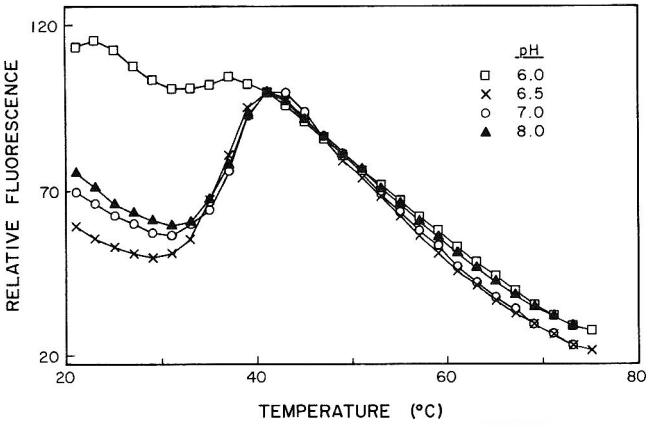

Fig. 4—Fluorescence intensity of tilapia myosin-ANS at 2.0 mg myosin/mL and 32 × 10⁻⁶M ANS. pH 6.0 (□), pH 6.5 (X), pH 7.0 (○), pH 8.0 (▲).

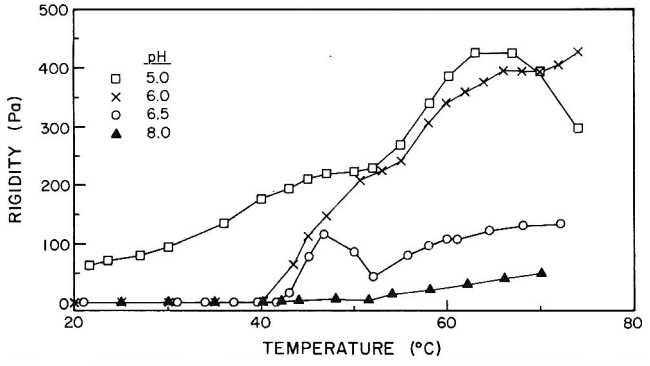

Fig. 5—TSRM thermograms of tilapia myosin at 8.1 mg/mL. pH 5.0 (□), pH 6.0 (X), pH 6.5 (○), pH 8.0 (▲).

gel were 36°C, 43°C, 43°C, and 42°C at pH 5.0, 6.0, 6.5, and 8.0, respectively.

KCl concentration effects on FI thermograms of fish myosin

Plots in Fig. 6 of FI vs temperature at different KCl concentration (pH 6.5) show that KCl concentration between 0.1M–

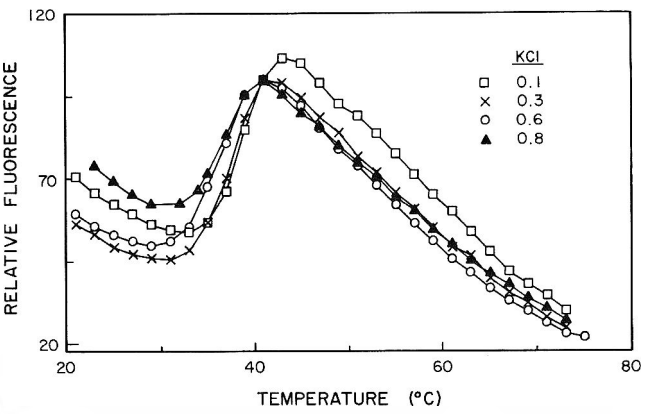

Fig. 6—Fluorescence intensity of tilapia myosin-ANS at 2.0 mg myosin/mL and 32 × 10⁻⁶M ANS. in 0.1M KCl (□), 0.3M KCl (X), 0.6M KCl (○), 0.8M KCl (▲).

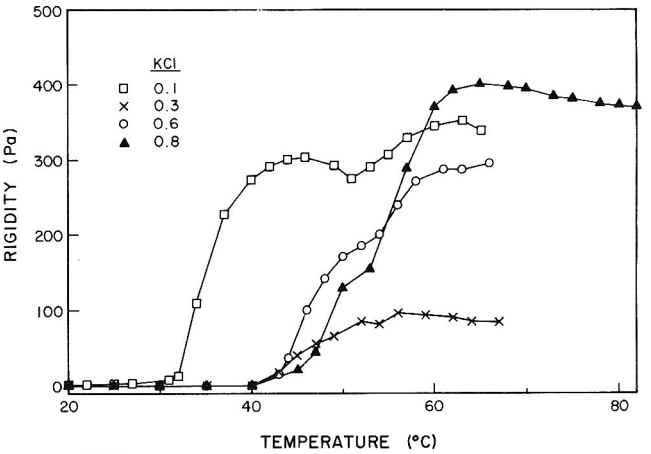

Fig. 7—TSRM thermograms of tilapia myosin at 6.3 mg/mL in 0.1M KCl (□), 0.3M KCl (X), 0.6M KCl (○), 0.8M KCl (▲). Values for 0.1M KCl are one tenth of the actual recorded values for the purpose of condensation.

0.8M did not influence the shape or magnitude of the fluorescence thermogram. First derivative plots of this data consistently yielded a Tr of 39°C. At 0.1M KCl, myosin existed as filaments and the sample was turbid; however, no change in the shape or magnitude of the thermal scans was observed in these samples despite the high level of turbidity.

KCl concentration effects on rigidity thermograms of fish myosin

The effect of KCl concentration at pH 6.5 on the rigidity thermograms of fish myosin during continuous heating is plotted in Fig. 7. In the soluble form, at KCl concentrations greater than 0.3M where myosin is primarily monomeric (Kaminer and Bell, 1966), the rigidity of myosin increased with increasing KCl concentration. Myosin at 0.8M KCl attained a higher final rigidity than myosin at 0.6M KCl and myosin at 0.3M KCl attained the least rigidity. At 0.1M KCl, fish myosin formed gels which attained a rigidity greater than 10 times that of the soluble myosin samples and gelation was initiated at a lower temperature. Gelation (increased rigidity) of myosin was initiated at 35°C, 43°C, 43°C, and 44°C in the presence of 0.1M, 0.3M, 0.6M, and 0.8M KCl, respectively.

The Tr of tilapia myosin-ANS thermograms conducted during the summer (Fig. 1) was slightly lower (37°C) than the Tr (39°C) of myosin-ANS fluorescence determined on tilapia myosin prepared during the winter months (Fig. 4,6). This difference in Tr values may be a seasonal phenomenon since identical preparative methods were used in each case.

Myosin-ANS FI was evaluated at myosin concentrations ranging 0.025–1.0 mg/mL and ANS concentration ranging 10^{-4} to 10^{-8} M. No effect of concentration on the Tr values was observed.

DISCUSSION

AS THE TEMPERATURE was increased, fluorescence decreased monotonically due to quenching. Therefore, any increase in FI of myosin-ANS complex must indicate a change in the hydrophobic character of the protein (Freifelder, 1982).

The increase in FI at 27°C of tilapia myosin-ANS (Fig. 1), therefore, indicates that myosin underwent a change in conformation over this temperature range. The temperature at which the FI began to decrease (41°C) closely paralleled the temperature at which the rigidity (at higher protein concentration) began to increase (41°C), suggesting that the decrease in FI at higher temperatures was not due solely to thermal quenching effects.

A similar increase and decrease in FI prior to the onset of gelation occurred in all species studied. As with tilapia myosin-ANS, the increase in FI of rabbit myosin-ANS and chicken myosin-ANS preceded the initiation of gelation in more concentrated solutions as measured by the TSRM. ANS measured a subtle change in the conformation of myosin involving hydrophobic residues during the thermal denaturation of myosin that was prerequisite to the gelation of myosin.

In a single preparation of myosin from Atlantic croaker (*Micropogon undulatus*), it was also observed (data not presented) that the temperature at which the FI of croaker myosin-ANS decreased paralleled the temperature at which the croaker myosin began to gel (about 37°C). Even though these results were observed from a single experiment, it is worthy to note that, in concentrated solutions of myosin of a species which is known to show marked "setting" (low temperature gelling) properties at 40°C (Lanier et al., 1982), a change in the hydrophobicity of myosin as measured with ANS was also observed just prior to initial increases in the rigidity.

The rigidity thermogram of tilapia myosin was similar in some respects to the thermogram observed in croaker surimi by Montejano et. al. (1984). These workers observed a distinctive peak near 40°C in the rigidity thermogram of croaker surimi which was thought to relate to the ability of croaker surimi to "set" or form a gel near 40°C. Similarly, the TSRM thermogram for tilapia myosin (Fig. 1) revealed a peak near 40°C, although tilapia surimi is known to gel more readily at 45°C than at 40°C (Shimizu et al., 1981).

The shape of the TSRM thermograms for rabbit myosin gelation is similar to those reported for rabbit myosin gelation as measured with a band type viscometer (Ishioroshi et al., 1979). However, these researchers reported two Tr values at 43°C and 55°C, whereas first derivative plots of the TSRM thermograms reveal a single Tr at 48°C. Furthermore, initiation of gelation was at 45°C as detected by the TSRM and at 35°C as detected by the band type viscometer. The explanation for such a discrepancy in Tr values may lie in the fact that the temperature at which a transition occurs depends not only on the temperature of the sample, but also on the rate of change of temperature in the sample. Montejano et al. (1984) noted that the TSRM transitions observed in croaker surimi were shifted to lower temperatures as the heating rate was decreased from 2.0°C/min to 0.5°C/min. As the heating rate decreased, transitions occurred at lower temperatures due to more heat absorption at a given temperature. Ishioroshi et al. (1979) and subsequent workers from their laboratory (Samejima et al., 1981; Ishioroshi et al., 1983) used isothermal incubations rather than a continuous heating rate. It is likely that the myosin molecule reached an equilibrium during the incubation period at 35°C and underwent changes in conformation which resulted in gel formation. If the heating rate during continuous rate measurements was sufficiently slow, much less than 1°C/min, it is probable that the observed Tr would be shifted downward to correspond more closely to that observed in isothermal measurements.

Neither the concentration of KCl nor pH greater than or equal to 6.5 affected fish myosin-ANS fluorescence thermograms, indicating that the ANS binding site was not influenced by electrostatic effects. The results at pH 6.0 (Fig. 4) are difficult to interpret. The lower Tr of 36°C at pH 6.0 suggested that myosin underwent a slight change in conformation at a lower temperature than occurred at higher pH values. However, pH 6.0 was close to the pI of myosin and the absence of a marked increase in FI may reflect a change in protein interaction without the involvement of the ANS binding sites.

The KCl concentration and pH did dramatically influence rigidity thermograms. The conditions which favor aggregation of myosin, such as low KCl concentrations and pH approaching the pI, were the conditions under which significantly more rigid gels were formed (Fig. 5,7). Ishioroshi et al. (1979) also reported that rabbit myosin at low KCl concentrations formed more rigid gels, although these sols during storage lost the ability to form gels sooner than myosin at higher KCl concentration. Myosin at KCl concentrations less than 0.3M KCl exists primarily as filaments. The number of monomeric species increases as the KCl concentration is increased (Kaminer and Bell, 1966; Weeds and Pope, 1977) and at pH values greater than pH 7.0 (Godfrey and Harrington, 1970a, b). Thus, the rigidity of myosin was increased by the same conditions which favored the aggregation of monomeric myosin units.

However, myosin gels at 0.1M KCl and pH 6.0 were much less elastic and less translucent than myosin gels formed at higher pH and KCl concentration. Ishioroshi et al. (1983) proposed that rabbit myosin formed thermally induced gels by a different process in low salt than in high salt. The extraordinarily high rigidity of myosin at 0.1M KCl and low pH may result from a coagulum type of denaturation which yields more opaque, brittle gels. At higher salt concentrations and pH values, the gels were more translucent and elastic indicating that denaturation led to a more orderly type of gelation. Gel elasticity is important to developing desirable texture in processed muscle foods.

CONCLUSIONS

ANS CAN BE USED as a probe for changes in myosin conformation and effective hydrophobicity during thermal denaturation and correlated with subsequent increases in rigidity of myosin from fish, rabbit and chicken. The increase in effective hydrophobicity preceded the onset of gelation, suggesting that the change in effective hydrophobicity was prerequisite for gelation. The gelation of myosin was also modified by electrostatic effects. The change in FI of myosin-ANS measured only changes in effective hydrophobicity, whereas the TSRM rigidity thermogram was a reflection of the total forces stabilizing a gel. The effect of KCl and pH on gel formation was dramatic and showed that aggregation of myosin filaments favored the formation of more rigid, opaque, and brittle gels. Under conditions which favored the monomeric form of myosin, such as high salt and alkaline pH, myosin formed a less rigid gel, which was more elastic and translucent.

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Diffusion of Glucose in Carrageenan Gels

M. HENDRICKX, C. VANDEN ABEELE, C. ENGELS, and P. TOBBACK

ABSTRACT

From an experimental set-up with boundary conditions of an extended initial distribution the diffusion coefficient for glucose in a high K-content carrageenan gel was evaluated as a function of the carrageenan concentration (1, 2 and 4%) and temperature (0.0, 5.0, 10.0, 15.0, 25.0, and 36.0°C). According to an Arrhenius-type equation, the activation energies at 1, 2 and 4% of carrageenan were calculated as 18.1, 17.4, and 19.1 kJ/mol. From these data it was concluded that carrageenan affects diffusion mainly by an obstruction effect.

INTRODUCTION

DIFFUSION PROCESSES IN gels play an important role in such technological applications as extraction, salting, drying, etc. . . (Schwartzberg et al., 1982; Stahl et al., 1979; Bichsel et al., 1976; Rodger et al., 1984; Fox, 1980; Bressan et al., 1981; Geurts et al., 1974; Belton et al., 1982). While diffusion in polymer gels is a topic of direct scientific interest on its own, gels also provide systems without free convection and it is often hoped that from such systems free solution diffusivities can be inferred (Muhr et al., 1982). Due to their gelling properties and their role as thickening agents carrageenans are widely used in the food industry. They are used in products such as desserts, puddings, sausages, pizza, etc. For this reason a study of the diffusivity of nutrients in carrageenan is of general interest. While the diffusivities of some components in gels have already been studied in some detail (Felicetta et al., 1949; White et al., 1961; Schantz et al., 1962; Ackers et al., 1962; Slade et al., 1966; Spacek et al., 1967; Li et al., 1968; Wong et al., 1971; Shaw et al., 1981; Nystrom et al., 1981; Muhr et al., 1982) much less information is available for carrageenan gels. The purpose of this study is to analyse the effect of carrageenan on the diffusion of a neutral water soluble molecule such as glucose.

THEORETICAL CONSIDERATIONS

THE DIFFUSION EQUATION of Fick in one dimension can be written as:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} \qquad (1)$$

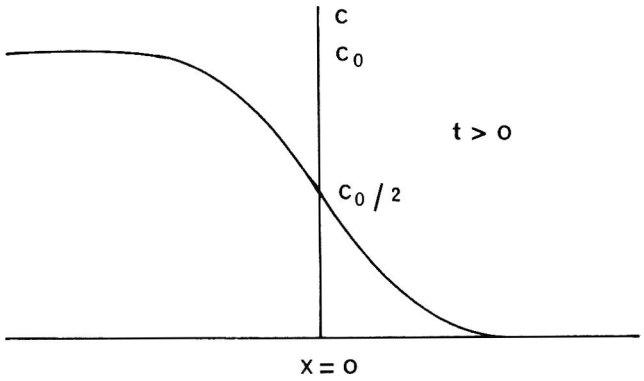
for diffusion in an isotropic medium with constant diffusion coefficient (independent of concentration). For an initial extended distribution (see Fig. 1) with the following initial- and boundary conditions:

$$C = C_o \quad x < 0$$
$$\text{for } t = 0$$
$$C = 0 \quad x > 0$$

the solution of Eq. (1) given by Crank (1970) provided the diffusion coefficient is constant:

$$C(x,t) = \frac{C_o}{2}\left[1\text{-erf}\left(\frac{x}{2\sqrt{Dt}}\right)\right] \qquad (2)$$

All authors are with the Laboratory of Food Technology, Catholic University of Leuven, Kardinaal Mercierlaan 92, 3030 Heverlee, Belgium.



(b)

Fig. 1—Concentration-distance curve for an initial extended distribution: (a) at time t = 0; (b) at time t > 0.

where C(x,t) is the concentration of the diffusant at distance x and time t.

The procedure to calculate the diffusion coefficient from the experimental data consists in (i) locating the X = O plane by use of Eq. (3) (an iterative search procedure using repeated linear interpolation and Simpson integration was used. The experimental concentration profile was scanned from the end of the diffusant-poor zone to the end of the diffusant-rich zone every 0.1 mm until the difference in surface changed sign), and (ii) evaluating the diffusion coefficient from the concentration distance curve according to Eq. (2).

$$\int_o^{C_o} x\,dC = 0 \qquad (3)$$

This method has been described previously by Naesens et al. (1981).

MATERIALS & METHODS

Diffusion set-up

The diffusion experiments were performed in thick-glass-wall cylinders of 1.2 cm inner diameter and about 7 cm length. Each cylinder was graduated at 1 mm accuracy. This system was described by Naesens et al. (1981). Approximately 2.5 cm of the cylinder, which was stoppered at one end, was filled with a hot carrageenan solution. This solution consisted of (U) g carrageenan (Genugel, type WG 103, high K-carrageenan content, A/S Kobenhavns Pektin-factory), (100-U) g distilled water and 70 mg sodium ethylmercury-thiosalicylate (Fluka) as a fungicide (U = 1, 2 and 4). Since the upper face of the sample was choosen as the (X = O)-plane, care was taken to make this face flat and orthogonal to the length-axis of the glass cylinder. The diffusant free layer was settled by cooling, then an equal amount of carrageenan solution containing the diffusant was poured on the top of the first layer. The total length of the diffusion profile was about 5 cm. To prevent mixing of the two layers the latter solution was cooled to a temperature just above the gelling point prior to introduction. One μci of C^{14}-glucose (D-U-C^{14}-glucose, Amersham International, Code CFB2) was added per 20 mL carrageenan solution. The whole system was then cooled at 5°C for a short time to gel the second solution, stoppered and incubated at the desired temperature in a horizontal position.

Table 1—Diffusion coefficient ($D \times 10^{10}$ m^2/s) of glucose at different temperatures and different carrageenan concentrations in a carrageenan gel (mean values for experiments in duplicate)

Temperature	Carrageenan concentration		
	1%	2%	4%
0	4.36	3.86	3.35
5	4.84	4.58	3.91
10	5.98	5.38	4.66
15	6.40	6.20	5.03
25	9.10	7.79	7.08
36	10.53	9.83	8.84

Concentration-distance curve

After a given period of time, the diffusion system was analyzed to obtain the concentration-distance curve. Diffusion times varied from 5 to 11 hr (those times were needed to get a well developed diffusion profile for different diffusion coefficients). The stoppers at both ends were removed and a plunger was gently introduced in the glass cylinder on the side of the diffusant-containing system. The plunger was moved forward by a screw action so that the gel system was gradually forced out of the cylinder. Slices of about 1.0 mm thickness were cut with a razor blade and each slide was collected in a pre-weighed plastic scintillation vial. The weight of each slice was determined and the activity was measured with a liquid scintillation counter (Beckmann LS 9000) using 0.5 mL water and 4.0 mL hydroluma (Lumac, 1077) as a scintillation medium. After addition of water and before adding the scintillation medium the vial was heated on a water-bath in order to destroy the gel and to get a homogeneous solution. No correction for quenching was found to be necessary and the background was subtracted. The activity of the last slices expelled from the system, expressed as cpm/g, was set equal to C_o and the relative concentration of the diffusant in the preceeding slices was calculated as C/C_o. When the activity of one of the five first slices, expelled from the diffusant-poor zone, was significantly higher than the background, the diffusion profile was rejected for further calculations. This was done to make sure that the last slices still had an activity of C_o. The concentration-distance curve could be easily calculated from the weights of the successive slices and the compression factor. Experiments were carried out in duplicate for gels of 1, 2 and 4% carrageenan at 0.0, 5.0, 10.0, 15.0, 25.0, and 36.0°C.

RESULTS & DISCUSSION

ALL DIFFUSION PROFILES were symmetric around the (X = O)-plane which was located according to Eq. (3). Diffusion coefficients were calculated on the basis of the least sum of squared deviations between the experimental concentrations C_e and the calculated concentrations C_c at the different locations. The C_c-values were calculated from equation (2) and the plane corresponding to X = O. This procedure was implemented on a computer using the NLIN-procedure from the SAS software package (SAS, 1982). The NLIN-procedure is based on the Gauss-method. The calculated diffusion coefficients at different temperatures and different carrageenan concentrations are summarized in Table 1. The mean square residuals for C/C_o varied from 0.00005 to 0.00034, no trends with distance were found.

The relationship between the diffusion coefficient and the carrageenan concentration was evaluated. A good fit was found for a linear relationship.

$$D = D_{oc} - a\,CAR \qquad (4)$$

Correlation coefficients using Eq. (4) at different temperatures are shown in Table 2, together with values for the diffusion coefficients for glucose extrapolated at 0% carrageenan according to Eq. (4). These extrapolated values always tend to be higher than those obtained with the diaphragm cell method as described by Longsworth et al. (1954) and Shaw et al. (1981) for the diffusion of glucose in water at various temperatures (see Table 2).

An Arrhenius type relation exists between the diffusion coefficient and the temperature:

$$D = D_o \exp(-E_A/RT) \qquad (5)$$

The results of a linear regression in a lnD versus 1/T plot are given in Table 3. This evaluation is graphically shown in Fig. 2. These data show that the activation energy is almost constant for concentrations of 0, 1, 2 and 4% carrageenan. The data obtained for 0% carrageenan correspond to the extrapolated values from Table 3.

The activation energy for the viscosity of water is 17.47 kJ/mol. This value was calculated from viscosity data as published by Weast et al. (1981). From the values of the activation energy for the viscosity of water and for the diffusion of glucose at all carrageenan concentrations it appears that the water present in the gel system behaves as free water. The good agreement between the activation energy for the diffusion of glucose in pure water (0% carrageenan) and the diffusion of glucose at all carrageenan concentration studied indicates that the carrageenan matrix acts as an inert obstacle during the diffusion of glucose. The same type of results and conclusions were reported by Brown et al. (1981) for the diffusion of a

Table 2—Diffusion coefficients for glucose extrapolated to 0% carrageenan concentration according to relation (4)

Temperature	r^2	$D_{oc} \times 10^{10}$ m^2/s	$D \times 10^{10}$ m^2/s (from literature)
0	0.99	4.62	
1	—	—	3.137[a]
4	—	—	3.500[b]
5	1.00	5.18	—
10	0.99	6.34	
13	—	—	4.736[a]
15	0.98	6.97	—
25	0.94	9.46	6.728[a]
36	1.00	11.02	
37	—	—	9.088[a]

[a] Data from Longsworth (1954)
[b] Data from Shaw and Schy (1981)

Table 3—Correlation coefficient and activation energy for diffusion of glucose in a 0, 1, 2 and 4% carrageenan gel

Carrageenan conc %	r^2	E_A
0	0.991	17.2[a]
	0.999[b]	21.0[b]
1	0.982	18.1
2	0.987	18.0
4	0.994	19.1

[a] Extrapolated values
[b] Literature values according to Chanal and Audran (1975).

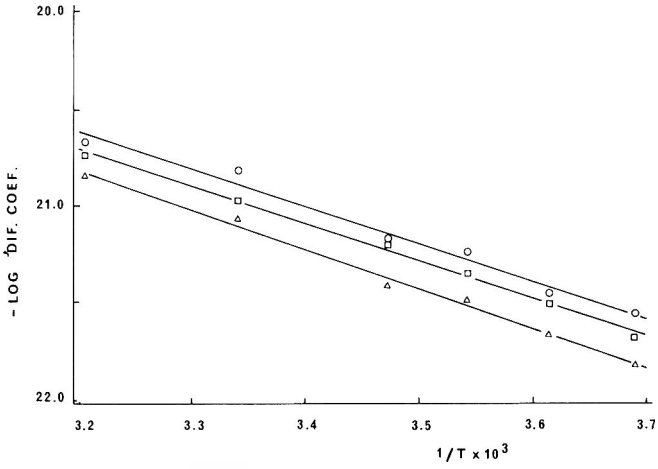


Fig. 2—The logarithm of the diffusion coefficient for glucose as a function of 1/T: Δ4.0% carrageenan; □ 2.0% carrageenan; ○ 1.0% carrageenan.

series of solutes in polyacrylamide gels. According to Brown et al. (1981) this means that carrageenan is affecting diffusion mainly as a result of its obstruction effect. This conclusion is in line with the work of Chanal et al. (1975) who studied the diffusion of H_2O and K^+ in a 5% K-carrageenan gel. They found an activation energy of 20.92 kJ/mol and 21.22 kJ/mol for the diffusion of K^+ and H_2O, respectively, listing an activation energy of 20.38 kJ/mol for the diffusion of water in pure water and gelatin gels.

For the diffusivity of solutes in gels the diffusion coefficient can be written as the diffusivity of the solute in the pure solvent multiplied by the volume fraction of the solution in the gel divided by the tortuosity. The partial volume of oligosaccharides is roughly 0.65 cm^3/g. For a 1% carrageenan gel the gel would occupy a fraction of 0.0065 of the volume and the surrounding solution would occupy a fraction of 0.9935 of the volume. For a 2% carrageenan gel these figures are 0.0131 and 0.9869, for a 4% carrageenan gel they are 0.0269 and 0.9731, respectively. The respective D-values are much smaller fractions of D_{oc} than 0.9935, 0.9869, and 0.9731. From Table 1 and Table 2 one can calculate 0.947, 0.855 and 0.752 at 1%, 2% and 4% carrageenan, respectively, as mean values for all temperatures. These data can be interpreted as 1g carrageenan associated with roughly 5 to 6g water which occupies space not available for diffusion. So it is the hydrated polymer that obstructs diffusion and the hydration region seems to be of more importance than the polymer itself.

CONCLUSIONS

FROM THE EXPERIMENTAL DATA it can be concluded: (1) an Arrhenius type equation exists between the diffusion coefficient for glucose in a carrageenan gel and the temperature, and (2) a linear relationship was found between the diffusion coefficient for glucose and the carrageenan concentration. After comparing activation energies for diffusion in water and diffusion in carrageenan gels it can be said that the hydrated polymer affects diffusion mainly because of its obstruction effect.

SYMBOLS

a	Constant
C	Concentration of the diffusant (mol/m^3)
C_e	Experimental relative concentration of the diffusant (C/C_o)
C_c	Calculated relative concentration of the diffusant (C/C_o)
C_o	Initial concentration of the diffusant (mol/m^3)
CAR	Carrageenan concentration (% carrageenan)
D	Diffusion coefficient (m^2/s)
D_o	Pre-exponential factor of the Arrhenius-equation (m^2/s)
D_{oc}	Diffusion coefficient at 0% carrageenan (m^2/s)
E_A	Activation energy (kJ/mol)
erf	Error function symbol
R	Universal gas constant ($8.314\ J.K^{-1}\cdot mol^{-1}$)
r^2	Correlation coefficient for linear regression
t	Time (s)
T	Absolute temperature (K)
x	Spacial coordinate (m)

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Enthalpy-Entropy Compensation in Sorption Phenomena: Application to the Prediction of the Effect of Temperature on Food Isotherms

R. J. AGUERRE, C. SUÁREZ, and P. E. VIOLLAZ

ABSTRACT

The isosteric heat and entropy of water sorption of food products were calculated from sorption isotherms previously reported by other researchers. The plot of the isosteric heat against entropy satisifed the enthalpy-entropy compensation. This relationship was used to derive a two-parameter sorption equation which took into account the effect of temperature on water sorption isotherms of food products.

INTRODUCTION

THE ENTHALPY-ENTROPY compensation has been widely investigated for different physical and chemical processes. One of the first papers in this area is by Bell (1937) who found a linear relation between the energy and entropy of solution for different solutes in the same solvent. Everett (1950b) investigated this relation for the adsorption of vapors on charcoal concluding that the entropy and the heat of adsorption were linearly related.

The parallelism between heat and entropy curves was observed by Schreiner and Kembal (1953) from measurements of adsorption isotherms of a number of vapors on oxides. However, the relation between magnitudes was not determined by these authors. The functionality enthalpy-entropy was calculated by Cremer (1955) for heterogeneous catalysis. This author introduced the term enthalpy-entropy compensation to designate the relationship between the two magnitudes.

A detailed analysis of the enthalpy-entropy compensation in organic chemical reactions was performed by Leffler (1955, 1966). Eighty-one examples of reactions were correlated by Leffler and Grunwald (1963), obtaining a linear relation between enthalpy and entropy, with a correlation coefficient equal to 0.979. The slope of this line has dimensions of absolute temperature and was designated by these authors as the "isokinetic temperature." They also demonstrated that when the isokinetic temperature was greater than the reaction temperature, the organic reactions were enthalpy controlled.

Labuza (1980) reviewed the enthalpy-entropy compensation in food systems and found that it applied well to the thermal death of microorganisms, protein denaturation and ascorbic acid degradation in several food systems. In a recent paper, Ferro Fontan et al. (1982) pointed out the existence of a linear reaction between enthalpy and entropy for water sorption in some foods. However, the authors did not analyze in detail this relationship and its implications in food sorption phenomena.

The purpose of this work was to analyze the enthalpy-entropy compensation for water sorption in different food products and present, on the basis of this relationship, a simple equation for relating equilibrium moisture, water activity and temperature.

The authors are with the Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria, 1428 Buenos Aires, Republica Argentina.

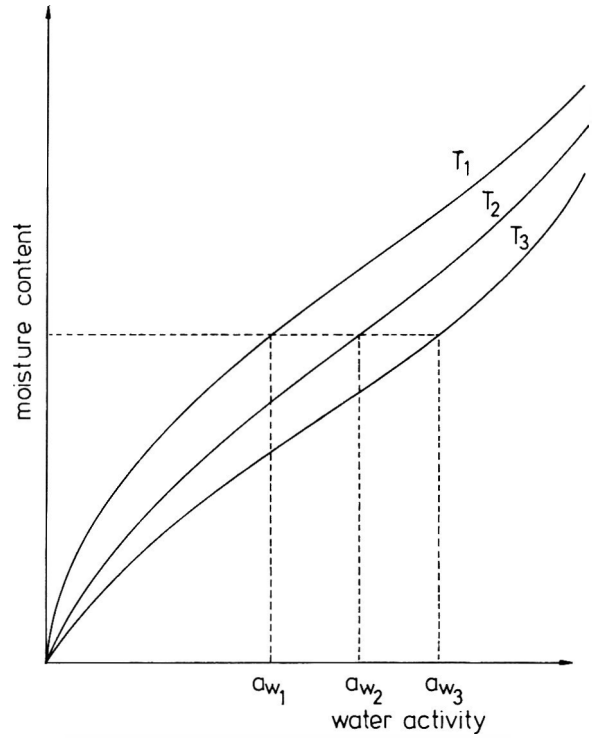

Fig. 1—Schematic representation of isotherm groups for the calculation of $\Delta H/\Delta S$ (T_1, T_2 and T_3 are temperatures).

MATERIALS & METHODS

EXPERIMENTAL water sorption data available in the literature were used to test the validity of the enthalpy-entropy compensation. The lack of sufficient data of sorption enthalpy-entropy makes necessary the estimation of both magnitudes, as will be shown later. The experimental isotherms selected were of those products for which equilibrium data at three or more temperatures were available to avoid excessive error in the determination of the enthalpy and entropy of sorption.

Values of enthalpy and entropy for water adsorption in four pectic products were taken from Bettelheim and Volman (1957). These pectic products were: pectin (I) (highly esterified pectin), sodium pectate (II) (enzymatically deesterified sodium pectate), sodium pectate (III) (alkali-deesterified sodium pectate), and pectic acid (IV) (pure pectic acid).

Experimental isotherms used

Values for hard red spring wheat at 25°C, 30°C, 35°C, and 50°C were taken from Day and Nelson (1965); for rough rice at 10°C, 20°C, 25°C, 30°C, and 40°C from Zuritz et al. (1979); for rough rice medium, at 40°C, 50°C, 60°C and 70°C from Aguerre et al. (1983); for anise seed, marjoram and thyme at 5°C, 25°C, and 45°C from Wolf et al. (1973); for corn at 4.4°C, 15.6°C, 30°C, 50°C, and 60°C from Hall and Rodriguez-Arias (1958); for mullet at 25°C, 43°C, and 54.5°C from Chau et al. (1982).

Calculation of the enthalpy and entropy of sorption

The isoteric heat and entropy of sorption were calculated from the equilibrium data given by the sorption isotherms. The relation between

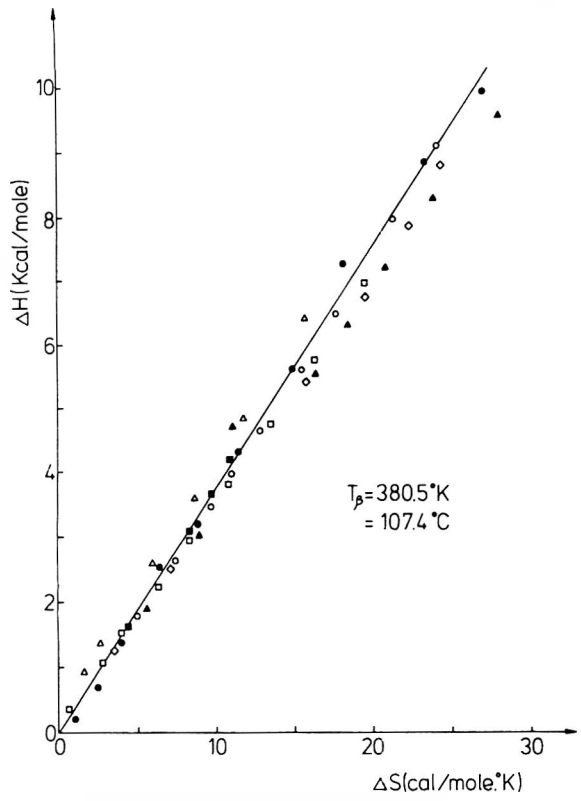

Fig. 2a—ΔH/ΔS relationship for water sorption in different foods and related products: ● wheat (Day and Nelson, 1965); ○ rough rice (Zuritz et al., 1979; Aguerre et al., 1983); △ anise seed (Wolf et al., 1973); ▲ mullet (Chau et al., 1982); □ marjoram (Wolf et al., 1973); ■ corn (Hall and Rodriguez-Arias, 1958); ◇ thyme (Wolf et al., 1973).

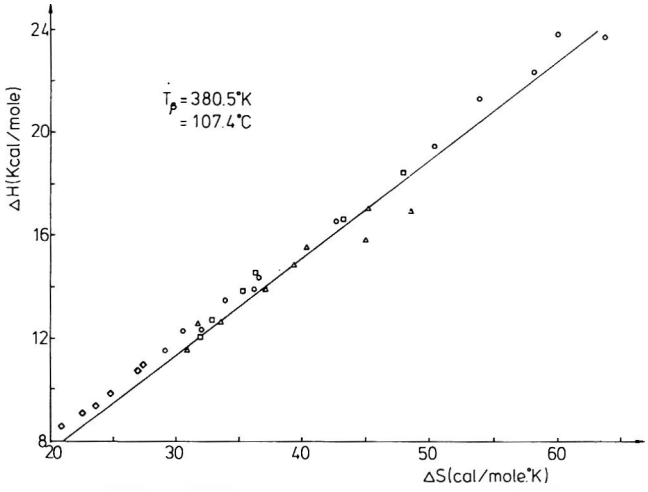

Fig. 2b—ΔH/ΔS relationship for water sorption in different foods and related products: □ pectin (I); △ sodium pectate (III); ○ sodium pectate (III); ◇ pectic acid (IV) (from Bettelheim and Volman, 1957).

the isosteric heat (ΔH) and differential entropy (ΔS) of sorption is given by the following equation (Everett, 1950a):

$$-\ln a_w = \Delta H/(RT) - \Delta S/R \quad (1)$$

where a_w is the water activity, T is the absolute temperature, and R the gas law constant. Eq. (1) can be used now to calculate ΔH and ΔS from the equilibrium data. At constant moisture, it can be seen from Fig. 1 that T is the only independent variable among the isostere. Hence, the a_w values corresponding to different temperatures must follow a straight line when plotted as $-\ln a_w$ vs 1/T, in accordance with Eq. (1). From this straight line the ΔH and ΔS values are easily

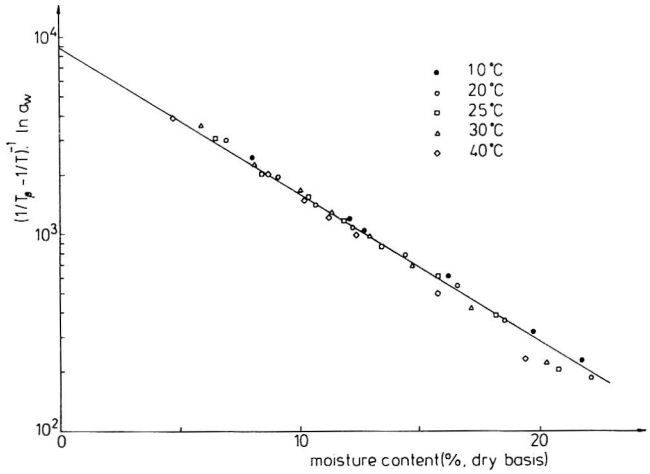

Fig. 3—Equilibrium data of rough rice at different temperatures plotted according to Eq. (5).

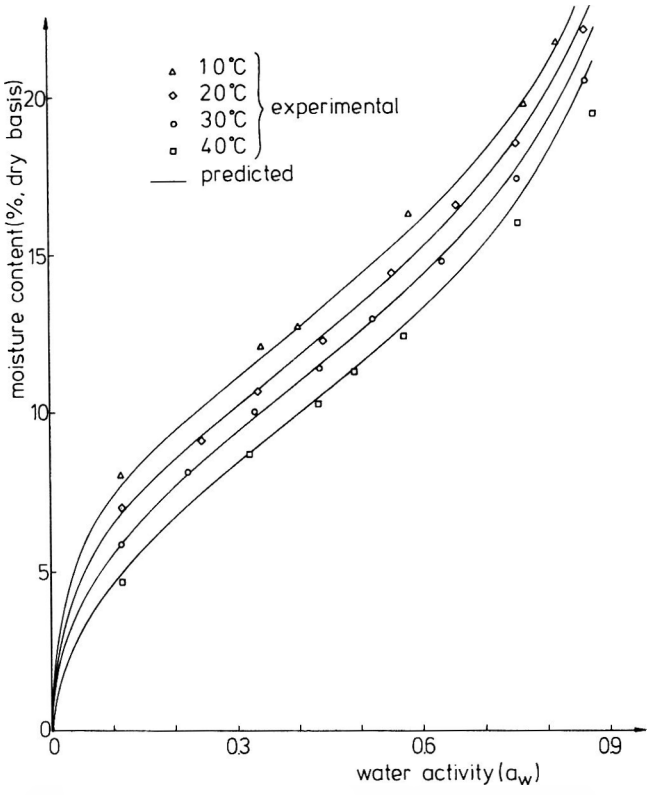

Fig. 4—Comparison between experimental data (Zuritz et al., 1979) and predicted isotherms from Eq. (6) (solid line) of rough rice at several temperatures.

calculated. This procedure can be repeated at different moistures to obtain the dependence of ΔH and ΔS with moisture.

RESULTS & DISCUSSION

THE ΔH AND ΔS VALUES corresponding to each of the products investigated were calculated by linear regression [according to Eq. (1)] from values of ln a_w vs 1/T, covering a range of water activity from approximately 4% to 80%. This procedure assumed that ΔH and ΔS did not vary with temperature or were essentially constant in the temperature range under consideration (Hakala, 1952). This hypothesis was confirmed in this study by calculating ΔH and ΔS values for a given product at different temperature intervals.

The ΔH and ΔS values of all selected products were correlated in accordance with Leffler and Grunwald (1963) in the

following form:

$$\Delta H = T_\beta \Delta S + \alpha \quad (2)$$

in which T_β is called the isokinetic temperature (Leffler, 1955) and α is a constant. The parameters T_β and α of Eq. (2) were calculated by linear regression from the data shown in Fig. 2-a and 2-b; $T_\beta = 380.5°K = 107.4°C$ and $\alpha = -0.087$ Kcal/mole. The good degree of correlation obtained ($r = 0.997$) between ΔH and ΔS, even with the diversity of the products selected, suggested that compensation existed. To corroborate this fact, the test of Krug et al. (1976a,b) was used. The authors suggested the comparison of T_β with the harmonic mean of the temperatures T_{hm}, defined as:

$$T_{hm} = \frac{n}{\sum_{1}^{n} (1/T)} \quad (3)$$

with n the total number of isotherms used. According to Krug et al. (1976a,b) the compensation only exists if $T_\beta \neq T_{hm}$. The value of T_{hm} corresponding to the present work is 305°K, which differs significantly from T_β.

The $\Delta H/\Delta S$ compensation will be used now to model the influence of temperature on the sorption isotherms. For this purpose Eqs. (1) and (2) can be combined to obtain:

$$-\ln a_w = \Delta H/R\ (1/T - 1/T_\beta) \quad (4)$$

In this derivation the parameter α of Eq. (2) has been neglected given its small contribution to the enthalpy change.

The functionality of water activity with moisture can be obtained in implicit form from Eq. (4) taking into account the dependence of the heat of sorption on moisture. This idea can be expressed by writing Eq. (4) in the form:

$$\Psi_T \ln a_w = K\, \emptyset(m) \quad (5)$$

where $\Psi_T = (1/T_\beta - 1/T)^{-1}$ and $\emptyset(m)$ is a certain empirical function of moisture, m.

One interesting aspect of Eq. (5) is that it provides a way of considering the influence of temperature on the sorption properties of a given system. In other words, it can be said that Ψ_T is the temperature correction factor in the isotherm given by Eq. (5). Therefore, it can be expected that if this factor is adequate, the equilibrium data of a given product, at different temperatures, will present the same functionality when they are plotted in accordance with this equation.

To test the validity of this assumption, the equilibrium data of rough rice reported by Zuritz et al. (1979) at several temperatures were plotted as $\ln (\Psi_T \ln a_w)$ against m in Fig. 3. In this case a good straight line was observed for the five temperatures reported by the authors. This result suggests that for the rough rice data reported by Zuritz et al. (1979) the dependence of $\Psi_T \ln a_w$ with m follows an exponential law of the form:

$$\Psi_T \ln a_w = K_1 K_2^m \quad (6)$$

The values of K_1 and K_2 calculated by linear regression are 8957°K and 0.842, respectively.

Eq. (6) is similar to the isotherm obtained by Bradley (1936) on the ground of thermodynamic considerations. Particularly, if T_β is much larger than T, Eq. (6) reduces to Bradley's equation. On the other hand, the value of K_2 found in this work agrees with the value of K_3 reported by Bradley (1936)for the adsorption of gases in inorganic substances (the value of K_3 reported by Bradley for type II sorption isotherms is nearly 0.8).

Eq. (6) was used to calculate a_w values at different moisture and temperatures. The results are shown in Fig. 4 for four temperatures, where the curves were calculated from Eq. (6) and the points are the equilibrium values reported by Zuritz et al. (1979). It can be seen that the agreement between predicted and experimental values was satisfactory.

An important aspect of Eq. (6), in comparison with other sorption equations available in the literature, was the small number of parameters required for describing the water sorption equilibrium at different temperatures. While Eq. (6) requires the determination of only two parameters, the sorption equations reported by other investigators (Chen and Clayton, 1971; Iglesias and Chirife, 1976) have four and three parameters, respectively. This simplicity is of practical importance, particularly for some engineering calculations where simplicity is required.

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Effectiveness of Sensory Difference Tests: Sequential Sensitivity Analysis for Liquid Food Stimuli

M. O'MAHONY and L. R. GOLDSTEIN

ABSTRACT

Versions of the triangle test in which either one or the other stimulus was maintained as the odd one, were found to differ in their effectiveness for a flavored sparkling water system but not for a wine system. The same was found for different orders of presentation of the R-index sensory difference test. These differences were predicted successfully from the signal detectabilities of the various stimuli using a predictive system called Sequential Sensitivity Analysis (SSA). This extended SSA beyond model systems to food systems. Use was made of a technique of rapid alternate tasting of different stimuli, called 'warm-up', to improve judges' performance.

INTRODUCTION

SENSORY DIFFERENCE TESTS play a vital part in the sensory evaluation of food. Yet, the performance of judges in difference tests varies with the particular procedure used; the reasons for this are not fully understood. It becomes essential to understand the physiological and psychological factors causing these differences so as to be able to control test sensitivity and develop more sensitive test procedures. Studies have been made for a variety of foods and model systems comparing the sensitivity of judges to differences, for different test protocols (Byer and Abrams, 1953; Dawson and Dochterman, 1951; Dawson et al., 1963; Filipello, 1956; Frijters, 1981; Gridgeman, 1955, 1956; Hogue and Briant, 1957; Hopkins and Gridgeman, 1955; O'Mahony et al., 1986; Pokorńy et al., 1981; Spencer 1979). For the triangle test (Helm and Trolle, 1946), differences within the triangular procedure have also been found, between versions where the odd sample has been the stronger or more familiar sample and where it has been the weaker or less familiar sample (Filipello, 1956; Frijters et al., 1982; Grim and Goldblith, 1965; Helm and Trolle, 1946; Hopkins, 1954; O'Mahony and Odbert, 1985; Pfaffmann et al., 1954; Wasserman and Talley, 1969). For the duo-trio test (Peryam and Swartz, 1950; Peryam, 1958) differences have been found between versions where the standard stimulus was the stronger of the two stimuli and where it was the weaker (Mitchell, 1956; O'Mahony and Odbert, 1985).

Various explanations have been invoked to explain these differences in effectiveness, ranging from sensory adaptation (Frijters, 1980; Grim and Goldblith, 1965) to sample variability (Helm and Trolle, 1946), guessing probabilities (Amerine et al., 1965) or stimulus search strategies (Pfaffmann et al., 1954). O'Mahony and Odbert (1985) proposed an explanatory model based on several interacting factors: sensory adaptation, stimulus confusion and differential sensitivity. The model considered how tasting a prior stimulus altered the signal detectability of a subsequent stimulus. For example, for two stimuli A and B, it considered the detectability of the second stimulus in each of the four possible paired sequences: AA, BA, BB, AB. Any order of tasting in a difference test can be seen to be made up of such sequence pairs. For example, the order of tasting in a triangle test, AAB, can be seen to be made up of two paired sequences: first AA, then AB. Likewise, all the possible orders of tasting for a given difference test can be broken down into sets of paired sequences. The signal detectabilities of the second stimuli in these paired sequences will indicate how well stimuli can be distinguished in that difference test. This analysis of the effectiveness of sensory difference testing protocols in terms of the detectabilities in sequence pairs was named Sequential Sensitivity Analysis (SSA).

SSA predicted successfully the relative effectiveness of various forms of the triangle and duo-trio tests. The present study extended the SSA model to liquid food systems for triangle and R-index (O'Mahony, 1979a, 1983) sensory difference tests.

The authors are with the Dept. Food Science Technology, Univ. of California, Davis, CA 95616.

MATERIALS & METHODS

Stimuli

The first set of stimuli comprised a test wine and the same test wine diluted by water. A 50: 50 mixture of a pre-blend and a full-blend Chablis was sweetened with 24 g sugar per L. To make the diluted wine, the test wine was diluted with water so that the ratio of wine:water was 85:15 by volume. Samples were dispensed in 10 mL volumes by Repipet Adjustable Dispenser (Labindustries, Berkeley, CA) and presented in 10mL pyrex beakers at constant room temperature (21–26°C), under red light to mask possible color differences. The Chablis wines were obtained from a California winery and refrigerated (1.7°C) until the day before testing when they were equilibrated to room temperature. C&H table sugar was used to sweeten the wine. The water was Millipore purified (Milli-RO 4/5 Filtration and Reverse Osmosis System, in series with a Milli-Q system: ion exchange and activated charcoal; Millipore Corp., Bedford, MA).

The second set of stimuli comprised a cherry flavored sparkling water and the same water sweetened by 0.0073gm sodium saccharin per L (equivalent to 0.03 mM). Approximately 10mL samples (10.45 ± 0.97 mL) were presented in 10mL pyrex beakers at constant room temperature (21-24°C). Cherry flavored Calistoga Sparkling water (Calistoga Mineral Water Co., Calistoga, CA) was refrigerated (1.7°C) until the day before testing when it was equilibrated to room temperature. Samples were prepared immediately before testing and sodium saccharin added as solution to preserve carbonation. Sodium saccharin was GPR grade (BDH Chemicals Limited, Poole, England).

Judges

Twelve judges (9M, 3F, age 20-29 yr) were sampled from students at UCD for the wine tasting and twelve (5M, 7F, age 20–29 yr; 4 from first sample) for the Calistoga water testing.

Procedure

The wine stimuli were tasted (sipping all the stimulus and expectorating) over two experimental sessions (second session 1-7 days after first; session lengths 1/2 to 1-1/2 hr). During these sessions, judges performed selected tests at their own speed, according to the scheme detailed in Table 1. Judges responded orally for all tests to an experimenter who was present throughout. Before each set of tests detailed in the table, judges rinsed the mouth with 150 mL purified water, otherwise mouthrinsing was omitted so as to highlight the effects of adaptation (O'Mahony, 1974, 1979b).

For the measurement of signal strengths for SSA, an adaptation of O'Mahony and Odbert's (1985) procedure was used. Judges first 'warmed-up' by tasting (and expectorating) alternately diluted and undiluted wine samples until they felt they could tell the difference (minimum, 3 or each stimuli; maximum 10). During 'warm-up', judges learned to call the diluted wine 'A' and the undiluted wine 'B', al-

Table 1—Order of presentation of testing procedures over the two experimental sessions

SESSION 1				
First part of 'warm-up' experiment.	First part of signal detectability measurement for SSA.	First part of comparison of triangle protocols.	Comparison of two presentation orders for R-index test.	Second part of signal detectability measurement for SSA.
9 triangles.	20 stimulus pairs.	12 triangles.	40 stimuli.	20 stimulus pairs.
SESSION 2				
Second part of 'warm-up' experiment.	Third part of signal detectability measurement for SSA.	Second part of comparison of triangle protocols.	Fourth part of signal detectability measurement for SSA.	Control to ensure effectiveness of masking red light.
9 triangles.	20 stimulus pairs	12 triangles.	20 stimulus pairs.	18 triangles.

though they were not told that the differences between A and B were due to dilution; no judges experienced difficulty with this task. As soon as this 'warm-up' procedure was finished, the judge proceeded with the experiment without pausing. Each judge tasted test samples of the two wines in random order. Immediately prior to tasting, expectorating and making a response for each test sample of wine, the judge was required to taste and expectorate a prior wine sample. The prior wine sample was either sample A or sample B, also given in random order. The prior wine sample was merely sipped and expectorated; the judge did not make a judgement about its taste. It was taken to alter the adaptation state of the taste receptors, immediately prior to tasting the test sample. The judge was not told whether the prior sample was A or B. The experimenter ensured that the test sample was sipped after the prior sample without pausing. Judges tasted 20 samples each of A after B, A after A, B after A and B after B. The total 80 stimulus pairs were presented in random order in blocks of 20, each with its own separate prior 'warm-up'; two blocks were given during each experimental session.

Judges were required to rate the stimuli as 'A-sure', 'A-not sure', 'B-sure', 'B-not sure'. From such ratings an R-index can be calculated (O'Mahony, 1979a, 1983). The R-index gives the detectability of each stimulus in terms of the probability of the judge distinguishing it better than the stimulus with the weakest detectability (undiluted wine tasted after undiluted wine). R-index signal strengths range from 50% (stimulus as distinguishable as the weakest stimulus: undiluted wine after undiluted wine) to 100% (stimulus always more distinguishable than the weakest stimulus). The signal strengths supplied the data for the SSA predictions regarding which forms of triangle and R-index sensory difference test were more effective.

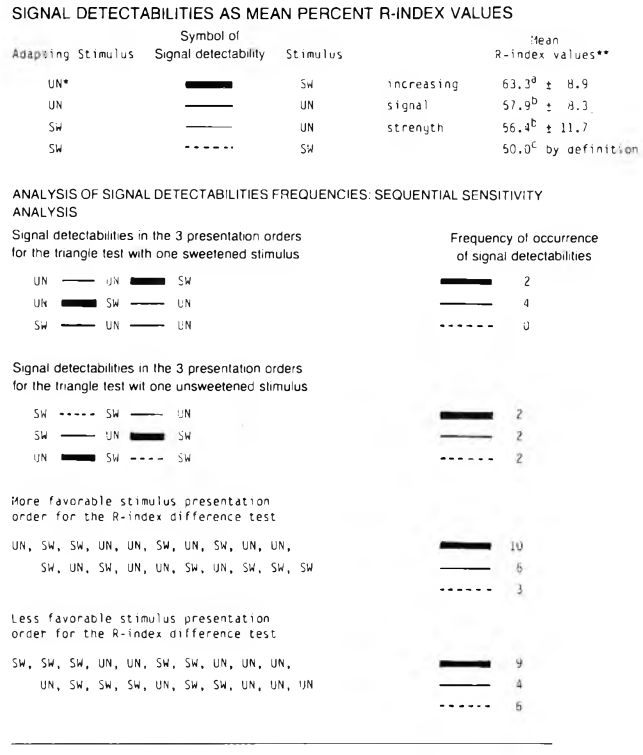
For a comparison of the two forms of the triangle test (diluted versus undiluted wine as the odd stimulus), judges first 'warmed-up' as before. Twenty-four triangles were given in random order so that the six possible stimulus orders of the triangle were tasted four times each. The 24 triangles were split over the two experimental sessions, each in a block of 12 with its own 'warm-up'. For all triangles, the judges were instructed to indicate the odd sample.

A comparison was also made for two possible orders of presentation for the R-index sensory difference rating test (O'Mahony, 1979a, 1983). A prior hypothetical SSA based on model systems (O'Mahony and Odbert, 1985), had indicated that one order might be more favorable than the other for effective differentiation between the stimuli. This test is not to be confused with the use of the R-index for measuring signal strengths for SSA.

For a given order, judges tasted 10 samples of diluted and 10 samples of undiluted wine in random order and rated the total 20 stimuli as 'A-sure', 'A-not sure', 'B-sure', 'B-not sure', according to the R-index rating protocol. R-index values were calculated giving the probability of distinguishing between A and B (ranging 50%: indistinguishable, to 100%: always distinguishable). The two orders of presentation were given directly after each other, counterbalanced over judges, during the first experimental session.

To study the efficacy of 'warm-up', each experimental session began with nine triangle tests, tasted either with or without a prior warm-up, in counterbalanced order over subjects.

To ensure that the red light was masking any possible color differences between wines, each judge performed 18 triangle tests in the second session (all six possible orders presented 3 times in random order; judge told either to identify darker or lighter color) by which they attempted to distinguish samples visually. The diluted samples were more dilute than those in the experiment (20% by volume) to make the task simpler and ensure the efficacy of the masking. All judges, except one, gave chance level performance (binomial $p > 0.39$). All data, from the one judge who performed better than chance, were dropped from further analysis since color differences may have contributed to his results.



*UN = Unsweetened cherry flavored Calistoga water sample; SW = Sweetened cherry flavored Calistoga water.

**Each mean ± standard deviation represents R-indices from 12 judges.

a,b,cMeans with different superscripts are significantly different (t test, $p < 0.05$).

Fig. 1—Signal detectabilities and sequential sensitivity analysis for cherry flavored Calistoga water stimuli, predicting the effectiveness of alternative forms of the triangle and the R-index sensory difference tests.

Table 2—Mean percent R-index values indicating the signal detectabilities for wine stimuli tasted immediately after various adapting stimuli

Adapting stimulus	Stimulus	Mean R-index values*
Undiluted wine	Diluted wine	56.2[a] ± 12.1
Diluted wine	Diluted wine	55.8[a] ± 15.0
Diluted wine	Undiluted wine	52.3[a] ± 6.2
Undiluted wine	Undiluted wine	50.0[a] by definition

* Each mean ± standard deviation represents R-indices from 11 judges.

[a] Means with the same superscripts are not significantly different ($p > 0.27$).

The same procedure was used for the cherry flavored Calistoga water as for the wine, except that the control for the efficacy of masking red light was unecessary and was omitted.

RESULTS & DISCUSSION

FIGURE 1 gives mean R-index values for the cherry flavored Calistoga water stimuli indicating relative detectabilites of each stimulus, depending on the stimulus that preceeded it. Table 2 gives the R-index values for the wines. The cherry flavored Calistoga water stimuli will be considered first. From the upper

part of Fig. 1, it can be seen that significant differences were found between the detectabilities. These detectabilities formed the basis for the SSA that predicted the relative effectiveness of the two versions of the triangle test and the two random orders for the R-index test. Considering the triangle tests, the numbers of strongest, weakest and intermediate detectabilities (denoted respectively by '▬', '—', '-----') can be computed from the stimulus orders. Clearly, the triangle test with one sweetened stimulus was weighted more in favor of stronger signal strengths than the triangle with one unsweetened stimulus and would thus be predicted to be more effective at distinguishing the two stimuli. Considering the two orders of presentation for the R-index test, the more favorable order can be seen to be weighted more towards better signal detectabilities and would thus be expected to be more effective.

Considering Table 2, it can be seen that for the wine stimuli, the detectabilities did not differ significantly, so, therefore, it would be predicted that there would not be differences in effectiveness between the two versions of the triangle and the two versions of the R-index test.

For cherry flavored Calistoga water, the mean number of triangle tests performed correctly by the judges, for the test with the odd sample sweetened (7.1, S.D. = 2.1) was greater than for the version with the odd sample unsweetened (5.1, S.D. = 2.6); this difference was sufficiently significant to confirm the SSA prediction (t test, $p < 0.06$). On the other hand, for the wines, the mean number of correct triangles indicated no significant differences (odd sample undiluted 5.0, S.D. = 1.0; odd sample diluted 4.6, S.D. = 2.1; t test, $p > 0.5$); this confirmed the SSA prediction.

For the R-index test, the mean percent R-index value for the more favorable order for cherry flavored Calistoga water (84.8, S.D. = 14.4) was greater than for the less favorable order (73.2, S.D. = 19.8); this difference was significant (t test; $p < 0.007$). For the wines, the means did not differ significantly (more favorable 77.6, S.D. = 14.0; less favorable 75.6, S.D. = 16.6; t test, $p > 0.39$). Again, SSA predictions were confirmed.

For the cherry flavored Calistoga water, definite trends were predicted by SSA for differences within the triangle and R-index protocols. Although mean trends are given here, it is worth noting that a significant majority of judges (10/12, binomial $p = 0.039$) followed the trends for their own individual SSA predictions for both triangle and R-index tests. For wines, such an analysis is inappropriate because the wines show no trend for differences in signal strengths and any differences from equality would be chance effects and not expected to show consistency for individual judges.

It is important to note that differences between test protocols depend on the particular food being tested. These determine the detectabilities of the stimuli in the test sequences. The relative detectabilities for the wine, and the cherry flavored Calistoga water vary and so do the SSA predictions for relative effectiveness of the test protocols. Because the SSA model takes this variation into account, it is thus a useful tool for matching the choice of suitable difference test to the stimuli at hand (ASTM, 1968).

Alternative explanations from the multi-faceted SSA model have been proposed to account for differences between the two alternative triangular designs. Adaptation, proposed by Frijters (1980) and Grim and Goldblith (1965) is included in the present model. Sample variability may have been a factor in Helm and Trolle's (1946) beer stimuli but here and in the previous study (O'Mahony and Odbert, 1985) controls ensured sample homogeneity. Pfaffmann et al. (1954) hypothesized that a triangle test with the odd stimulus constant would be easier than the traditional protocol using both stimuli as the odd sample, because the judge need only search for a single signal. Amerine et al. (1965) argued that it would be easier because if the judge learned or was told that the odd sample was always the same, the test would be reduced to a paired test, since any one comparison would establish the odd sample; with $p = 1/2$ this would give a greater degree of success. However, O'Mahony and Odbert (1985) demonstrated how SSA correctly predicted that the traditional triangle test protocol was intermediate in effectiveness between the protocol with the strong stimulus odd and the one with weak stimulus odd.

Use was made in this study of 'warm-up'. 'Warm-up' here refers to the phenomenon whereby after repeated alternate tasting of two stimuli, the judge suddenly becomes more able to distinguish between the two. At first, the difference between the stimuli cannot be perceived and then the sensation signalling the difference suddenly appears. To the judge, the sudden appearance of the signal resembles the sudden appearance of the sound after 'warming-up' an old fashioned pre-transistor radio; this accounts for the phenomenon being called 'warm-up'. It is not the same as 'practice' which refers to a gradual (not a sudden) improvement in performance, over periods of time longer than the few minutes required for 'warm-up'. The 'warm-up' phenomenon has been variously reported for taste (Linker et al., 1964; O'Mahony, 1972; O'Mahony and Odbert, 1985; O'Mahony et al., 1984, 1986; Peryam and Swartz, 1950; Pfaffmann et al., 1954). Although the mechanism of 'warm-up' is not understood, it may be hypothesized that when 'warming-up' a judge is attempting to focus on the differences between the two stimuli; 'warm-up' is the result of a successful signal search. 'Warm-up' was used in the preliminary stages of these experiments to eliminate the effects of stimulus unfamiliarity, which could cause judges to be unsuccessful in earlier parts of the experimental sessions because they had not received sufficient input to be completely familiar with the sensory signals elicited by the stimuli. The use of 'warm-up' ensured that differences in performance of the judges were due to test protocols rather than judges' confusion.

Although 'warm-up' is an effect that has been reported subjectively, it has not been studied systematically. Here, the mean number of correct triangle tests performed after 'warm-up' (Calistoga water 5.1, S.D. = 1.7; wine 5.3, S.D. = 1.5) was greater than the mean number after no 'warm-up' (Calistoga water 3.3, S.D. = 0.9; Wine 3.3, S.D. = 1.3); these differences were significant (t test; $p \leq 0.02$). This demonstrates the efficacy of 'warm-up' in increasing the judges' effectiveness. The technique has potential in sensory evaluation.

It is worth noting that the terms 'triangle test' and '3-AFC (3-alternative forced choice) test' are applied to tristimulus testing designs. Generally, the name 'triangle test' is used when all six orders of presentation are given (AAB, ABA, BAA and BBA, BAB, ABB) and the instructions are to indicate the odd stimulus without specification of the nature of the oddity (Amerine et al., 1965; Frijters et al., 1980). The 3-AFC test is an example of the more general m-AFC procedures (Green and Swets, 1966). Here, only one of the sets of triads are presented (AAB, ABA, BAA or BBA, BAB, ABB) and the instructions are directed; the judge is told to identify the stimulus containing the signal. This would seem to imply some knowledge of the nature of the oddity. Frijters et al. (1980) implied that in this case the judge would be asked to identify the stronger (or weaker) sample, not merely to identify the odd sample. However, from Green and Swets' (1966) definition of m-AFC tests, 'finding the signal' could equally well apply to either instruction. In the present study, with instructions to indicate the odd stimulus, we have referred to the alternative forms as triangle tests; they could also be referred to as 3-AFCs (O'Mahony and Odbert, 1985).

CONCLUSIONS

SSA can be successfully extended beyond model stimuli to predict from relative signal detectabilities, the effectiveness of alternative forms of triangle test and orders of presentation in the R-index difference test. The relative signal strengths varied with the food system as did the predictions, which were con-

firmed. The SSA predictive model for the relative effectiveness of difference testing protocols has the advantage of altering its predictions to take into account differences between food systems. The model would seem to be useful in understanding the efficacy of various difference testing protocols and an aid to the development of new ones. A technique with the potential for increasing the effectiveness of difference tests is 'warm-up'; it is worthy of further investigation.

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A Research Note

A Simplified Myosin Preparation from Marine Fish Species

CELINA B. MARTONE, LILIANA BUSCONI, EDUARDO J. FOLCO, RAÚL E. TRUCCO, and JORGE J. SANCHEZ

ABSTRACT

A simple and rapid method for the purification of fish myosin with high yield was developed. The method consisted of washing chopped fish muscle with a 0.1M KCl solution, an extraction of myosin with a 0.45M KCl solution containing ATP, and steps of reprecipitation and redissolution by changes in ionic strength. Actin can easily be obtained as a component of the myosin preparation.

INTRODUCTION

IT IS WELL KNOWN that the myofibrillar proteins are essential for maintaining the binding properties of comminuted meat and fish products, such as sausages and kamaboko. Since these proteins are also partially responsible for fat and water-binding capacities and emulsifying ability, their alteration could have an adverse effect on the physical properties of the final products. Myosin, the major constituent of myofibrils, is of technological significance, due to its participation in the rheological changes that take place during the storage and processing of meat and fish products (Yamamoto et al., 1977; Yasui et al., 1980; Lin and Lanier, 1981; Rodger et al., 1984).

The preparation of myosin from marine fish species was undertaken to achieve simple, isolated systems for studying the participation of muscle proteases in the deterioration of fish products. Several methods previously reported (Connell, 1960; Hasnain et al., 1979; Kimura et al., 1980) were attempted, but impure preparations or low yields were obtained. The purpose of this study was to develop a new method for the preparation of myosin with high yield and purity from both lean and fatty fish species.

MATERIALS & METHODS

MUSCLES from *Merluccius hubbsi* (hake), *Micropogon opercularis* (white croaker), *Engraulis anchoita* (anchovy), *Pagrus pagrus, Acanthistius brasilianus, Parona signata* and *Cynoscion striatus* were used. Fish were captured by commercial vessels, stored on ice, transported to the laboratory within 2–4 days and used immediately. All samples were in the post-rigor stage.

All chemicals were reagent grade. ATP and molecular weight standards were from Sigma Chemical Co. (St. Louis, MO). Protein concentration was determined by the method of Lowry (Layne, 1957) using bovine serum albumin as a standard. Inorganic phosphate was determined by the method of Chen et al. (1956). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Porzio and Pearson (1977). Fifty micrograms protein were loaded on each gel. Molecular weights were determined using rabbit myosin (200,000), cross-linked bovine albumin (264,000; 198,000; 132,000 and 66,000), ovalbumin (45,000), pepsin (34,700), trypsinogen (24,000), soya-bean trypsin inhibitor (21,500) and lysozyme (14,300) as standards.

The authors are affiliated with Instituto Nacional de Tecnología Industrial, INTI, Centro de Investigaciones de Tecnología Pesquera, CITEP, Marcelo T. de Alvear 1168. 7600–Mar del Plata, Argentina. Author Trucco is Career Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina). Author Martone is affiliated with Consejo Nacional de Investigaciones Científicas y Técnicas.

All the steps in the preparation of myosin were carried out at 0–4°C. Special care was taken in avoiding the formation of foam all through the process, since myosin is very sensitive to denaturation in liquid-air interphases (Pollard, 1982a). The composition of the solutions used in the preparation was as follows: soln. A: 0.10M KCl, 1mM phenylmethylsulfonylfluoride (PMSF), 0.02% NaN_3 and 20 mM Tris-HCl buffer pH 7.5; soln. B: 0.45M KCl, 5 mM β-mercaptoethanol (β-MCE), 0.2M $Mg(CH_3COO)_2$, 1 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), 20 mM Tris-maleate buffer pH 6.8; soln. C: 0.50M KCl, 5 mM β-MCE, 20 mM Tris-HCl buffer pH 7.5; soln. D: 0.80M KCl, 5 mM β-MCE, 0.2M Mg $(CH_3COO)_2$.

For adenosine 5′-triphosphatase (ATPase) determinations, the following incubation mixtures were chosen: Ca^{2+}-ATPase: 5 mM $CaCl_2$, 100 mM KCl, 5 mM β-MCE, 20 mM Tris-HCl buffer pH 7.5; ethylenediaminetetraacetic acid (EDTA)/ATPase: 2mM EDTA, 0.8M KCl, 5 mM β-MCE, 20 mM Tris-HCl buffer pH 7.5; Mg^{2+}-ATPase: 0.05M KCl, 0.1 mM $CaCl_2$, 2 mM MgCl, 5 mM β-MCE, 20 mM Tris-HCl buffer pH 7.5. One hundred fifty to three hundred micrograms myosin per mL of incubation mixture were used. Mixtures were incubated at 30°C for 10 min, and the reactions were stopped by addition of equal volume of cold 10% trichloroacetic acid solution. The mixtures were centrifuged at 2,000 × *g* for 15 min; inorganic phosphate (Pi) was determined in the supernatants. When the effect of actin was studied, the protein was added at the beginning of incubation in the proportion indicated in each case.

RESULTS & DISCUSSION

THE FLOW DIAGRAM of the procedure is represented in Fig. 1. In step 1, the disintegration of muscle produced a gelatinous material that hindered extraction. Hamoir et al. (1960) observed a similar behavior in carp and mullet white muscles. In step 5, holding the mixture for up to 2 hr at 0°C did not increase the amount of myosin extracted.

The pellet 9 contained undissociated actomyosin and Mg-actin (Kimura et al., 1980). Actin could be recovered from it by following the modification of the method of Ebashi (1976) (Fig 1).

Concentrations of 10–15 mg/mL and 0.5–0.7 mg/mL were obtained for myosin and actin, respectively. Electrophoretic patterns of actin from hake skeletal muscle and myosin from seven different fish species are shown in Fig. 2. Molecular weights of 200,000 and 40,000 were obtained for the myosin heavy chain and actin, respectively. Two bands corresponding to polypeptides with molecular weights of 22,000–24,000 (weak) and 16,000–19,000 (strong) were obtained in the zone of myosin light chains. The former presumably corresponded to light chain 1, while the latter could be a mixture of light chains 2 and 3, which were not properly resolved in our electrophoretic system.

The degree of purity of myosin and actin preparations was higher than 95% as judged by densitometric analysis of the SDS-polyacrylamide gels. Moreover, the ratio of optical density at 280 nm to that at 260 nm (O.D. 280/O.D. 260 > 1.64) in myosin preparation indicated that contamination with nucleic acids was lower than 0.25% (Layne, 1957; Buttkus, 1966).

The functionality of myosin was checked by measuring the activity of Ca^{2+}- and EDTA-ATPases, both typical of myosin, and Mg^{2+}-ATPase, characteristic of actomyosin. White croaker myosin showed a low Mg^{2+}-ATPase activity (0.008 μmol Pi

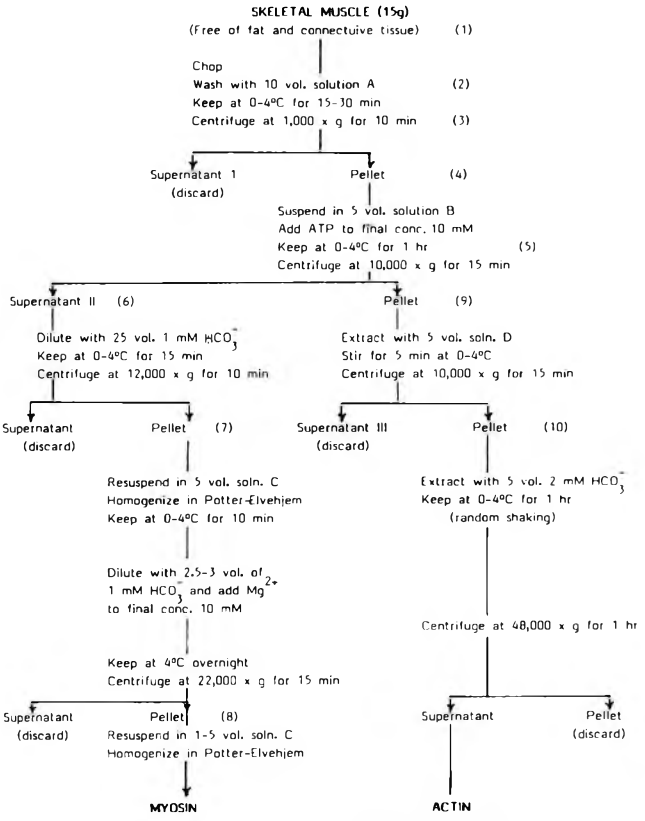

Fig. 1—Flow diagram for the preparation of myosin and actin.

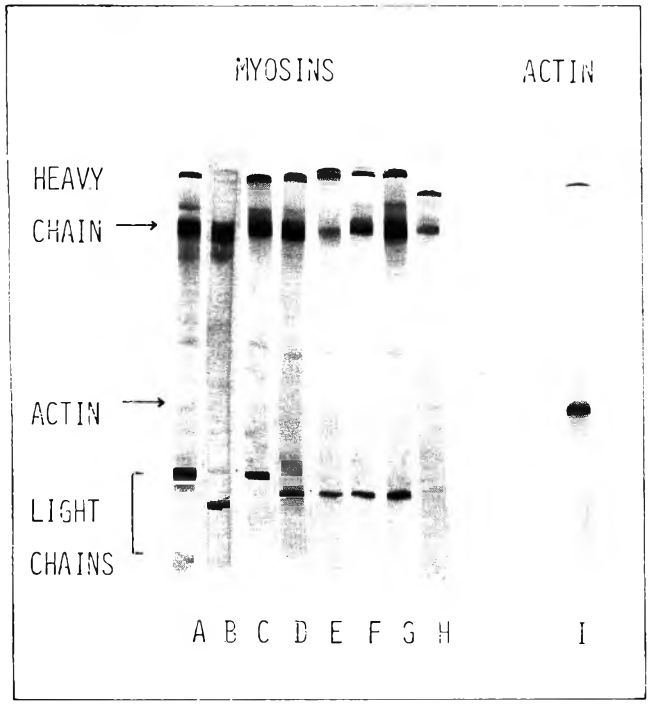

Fig. 2—SDS-PAGE patterns of myosins (A-G) and actin (I) from different marine fish species. (A) and (I) Merluccius hubbsi; (B) *Micropogon opercularis;* (C) *Engraulis anchoita;* (D) *Pagrus pagrus;* (E) *Acanthistius brasilianus;* (F) *Parona signata;* (G) *Cynoscion striatus;* (H) *Rabbit myosin* (from Sigma Chemical Co.).

$min^{-1} \cdot mg^{-1}$) that was strongly activated (260%) when actin was added to an actin/myosin ratio of 0.4(w/w), which indicated that myosin was not contaminated with actin (Pollard, 1982b).

Specific activity values of 0.30–0.40 μmol Pi $min^{-1} \cdot mg^{-1}$, pH optima of 6.8 an 8.5 and a K^+ concentration optimum of 0.1M were obtained for Ca^{2+}-ATPase, while specific activity values of 0.20–0.24 μmol Pi $min^{-1} \cdot mg^{-1}$ and a K^+ concentration optimum of 0.8–1.0M were obtained for EDTA-ATPase. These values are similar to those reported for myosins from trout (Buttkus, 1966), carp (Hasnain et al., 1979), squid (Kimura et al., 1980) and rabbit (Sigma Chemical Co.) muscles. Actin showed a strong inhibitory effect (about 90%) on both Ca^{2+}- and EDTA-ATPases when it was added to myosin at a ratio of 0.25–0.40 (w/w). These values were obtained with myosin and actin purified from fish stored on ice for 2–4 days. Despite some denaturation that could have occurred during the storage these results indicated that both myosin and actin were obtained as functional proteins.

Myosin can be stored in 50% glycerol at −15°C for at least 2 months without changing its electrophoretic pattern and its capacity as substrate for fish muscle proteases (Folco et al., 1984). Sixty to eighty percent of its initial Ca^{2+}-ATPase activity was retained after 10 days of storage (measurements after longer periods were not performed).

Myosin was obtained with a yield of 2% on wet tissue basis, which corresponded to about 25% of total muscle myosin. This yield and the concentration obtained by this method are higher than those reported by Connell (1960) for cod (yield 1.1–1.4%, concentration 0.5%), Hamoir et al. (1960) for carp (yield 0.3–1.0%, concentration 0.4%) and Focant and Huriaux (1976) for carp (yield 1.6%) and pike (yield 0.8%) skeletal muscles. Furthermore, the technique used in this preparation was simpler, and the time employed was shorter than in those above mentioned.

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Dedicated to Dr. Luis Federico Leloir on the occasion of his 80th birthday, Sept. 6, 1986.

A Research Note

Differences in the ω3 Fatty Acid Contents in Pond-Reared and Wild Fish and Shellfish

P. CHANMUGAM, M. BOUDREAU, and D.H. HWANG

ABSTRACT

The fatty acid composition of total lipids from edible portions of pond-reared prawn, catfish, and crayfish were compared with those of their wild counterparts. It was found that the lipids of the cultured animals had higher levels of ω6 fatty acids and lower ω3 fatty acid levels and ω3/ω6 ratios compared with their wild counterparts. The pelleted catfish diet was rich in ω6 fatty acids. It was concluded that the lipids of pond-reared fish and shellfish may not have the high levels of ω3 fatty acids found in wild seafood, and that the feasibility of increasing the ω3 fatty acid content by dietary manipulation, needs to be investigated.

INTRODUCTION

EPIDEMIOLOGICAL, clinical, and biochemical studies performed during the last ten to fifteen years have suggested that consumption of seafood is beneficial in reducing the risk of coronary disese (Dyerberg, 1986; Glomset, 1985; Herald and Kinsella, 1986), which is the leading cause of death in the United States. This beneficial effect is considered to be primarily due to the lipid in marine animals which is rich in polyunsaturated fatty acids (PUFA) of the ω3 family, particularly eicosapentaenoic acid (EPA). Most vegetable oils and the lipids of domestic animals are rich in ω6 fatty acids with arachidonic acid (AA) being the major 20 carbon PUFA. These findings could lead to an increased demand for both wild and cultured seafood.

The culturing of certain fish and shellfish has become a profitable business in some parts of the United States (Hanson and Godwin, 1977); however, Ackman (1974) has pointed out that cultivated fish may have high levels of ω6 fatty acids, reflecting their vegetable oil based diets. In a previous study (Chanmugam et al., 1983), it was found that the fatty acid composition of lipids from whole, freshwater prawn resembled lipids from terrestrial animals with high levels of ω6 fatty acids rather than marine animals. Cultured fish are often fed diets based on soybean meal in which ω6 fatty acids predominate, whereas marine fish consume diets based on plankton, which is rich in ω3 fatty acids (Ackman, 1982).

As the general public becomes more aware of the health benefits of seafood consumption, the nutritional quality of aquaculture products could become one of the factors in determining consumer acceptance in the future. It will therefore be important to determine if there are differences in the ω3 fatty acid content of wild and pond-reared aquatic animals. This study compares the fatty acid composition of lipids from edible portions of pond-reared freshwater prawn, catfish, and crayfish with those from their wild counterparts.

MATERIALS & METHODS

POND-REARED FRESHWATER PRAWN, crayfish, and channel catfish were obtained from the Louisiana State University Experiment Station Acquaculture Farm, and Gulf shrimp, wild channel catfish, and basin crayfish were obtained from LSU Cooperative Extension County agents. Samples were frozen immediately after collection and kept frozen until assayed. Total lipids were extracted from edible portions (shelled prawn, shrimp and crayfish tails and catfish tail muscle) of the seafood as well as the commercial catfish diet by the method of Folch et al. (1957) and quantitated by the AOAC (1970) method. Total lipids were saponified and methylated (Morrison and Smith, 1964) and fatty acid composition was determined by gas liquid chromatography as described previously (Hwang and Carroll, 1980). Individual fatty acids were identified using fatty acid standard mixtures from Supelco (Bellefont, PA). Duncan's multiple range test was used for statistical analysis of the data.

The authors are with the Louisiana Agricultural Experiment Station, Human Nutrition and Foods, Home Economics Bldg., Louisiana State Univ., Baton Rough, LA 70803.

RESULTS

THE TOTAL LIPID contents (g/100g) of the edible portions of shrimp and prawn were not significantly different (1.00 ± 0.56 vs 1.08 ± 0.11). Previous work (Chanmugam et al., 1983) showed that the lipid content of whole prawn was almost three times greater than that of whole marine shrimp. This implies that the greater amount of lipid accumulated by the prawn is not associated with the tail muscle. The fatty acid composition of total lipids from shrimp and prawn tails are listed in Table 1. There were no differences in total saturated and total monounsaturated fatty acids or total PUFA, but total ω6 PUFA was significantly greater ($P < 0.5$) in prawn, primarily due to higher levels of linoleic acid (18:2ω6). Total ω3 PUFA and the ω3/ω6 ratio were significantly lower ($P < 0.05$) in prawn compared to shrimp.

There was no difference in total lipid content (g/100g) of the edible portions of wild and pond-reared crayfish (0.59 ± 0.06 vs. 0.59 ± 0.03). The fatty acid composition of total lipids from crayfish tails are listed in Table 1. There were no differences in total saturated and monounsaturated fatty acids, total PUFA or total ω6 PUFA between wild and pond-reared crayfish. Total ω3 PUFA and the ω3/ω6 ratio was lower in pond-reared crayfish compared to the wild crayfish, but the differences were not statistically significant.

The total lipid content (g/100g) was significantly greater ($P < 0.05$) in edible portions of cultured catfish compared to wild catfish (3.03 ± 0.19 vs. 0.47 ± 0.26), indicating that in these fish excess lipids accumulate in the tail muscle. However, Bonnet et al. (1974) reported a slightly higher fat content in the edible flesh of wild catfish compared to cultured catfish (1.80 ± 0.21 vs 1.48 ± 0.18).

The fatty acid composition of total lipids from edible portions of wild and pond-reared catfish as well as the catfish diet are listed in Table 2. There are considerable differences between pond-reared and wild catfish, particularly in levels of linoleic acid (18:2ω6) eicosapentaenoic acid (20:5ω3) and docosahexaenoic acid (22:6ω3). Total ω6 PUFA was significantly higher ($P < 0.05$) and total PUFA, total ω3 PUFA and the ω3/ω6 ratio significantly lower ($P < 0.05$) in pond-reared catfish compared to wild catfish. However, the level of arachidonic acid in wild catfish was higher than that of pond-reared catfish.

DISCUSSION

THE CULTURED CATFISH were reared in ponds and fed a commercial pelleted catfish diet. The prawn were reared in the same ponds and probably consumed some of the catfish diet. The crayfish were raised in rice-planted ponds from which they derived their food and were not fed any supplemental diet.

Table 1—Fatty acid composition of total lipids from edible portions of marine shrimp, pond-reared prawn and crayfish[a]

Fatty acid	Marine shrimp	Pond-reared prawn	Crayfish	
			Wild	Pond-reared
16:0	21.24 ± 1.42	23.52 ± 0.79	19.50 ± 1.54	17.08 ± 0.35
16:1ω7	8.80 ± 0.28	7.21 ± 0.23*	5.78 ± 0.30	6.07 ± 0.46
18:0	11.16 ± 0.22	7.93 ± 0.95*	6.99 ± 0.34	7.78 ± 0.38
18:1ω9	13.51 ± 0.59	19.34 ± 0.49*	17.56 ± 0.65	21.08 ± 0.44
18:2ω6	5.99 ± 0.53	12.04 ± 0.21*	7.15 ± 0.26	8.05 ± 0.46
20:1ω9	0.28 ± 0.16	0.35 ± 0.12	—	—
18:3ω3	5.13 ± 0.70	2.63 ± 0.19*	7.54 ± 0.61	4.72 ± 0.23†
18:4ω3	0.74 ± 0.08	0.79 ± 0.05	0.90 ± 0.05	1.19 ± 0.04†
20:3ω6[b]	0.59 ± 0.10	0.23 ± 0.04	0.23 ± 0.06	0.25 ± 0.04
20:4ω6	8.98 ± 0.65	9.75 ± 0.47	7.98 ± 0.15	7.55 ± 0.20
20:4ω3	0.27 ± 0.03	0.93 ± 0.36	0.42 ± 0.13	0.14 ± 0.05
20:5ω3	13.94 ± 0.58	10.77 ± 0.37*	18.32 ± 0.73	20.18 ± 0.65†
24:1ω9	0.03 ± 0.00	0.35 ± 0.06	0.18 ± 0.10	0.19 ± 0.04
22:4ω6	0.54 ± 0.15	0.36 ± 0.04	0.37 ± 0.13	0.57 ± 0.12
22:5ω6	0.98 ± 0.21	0.67 ± 0.14	0.66 ± 0.17	0.23 ± 0.09
22:5ω3	0.97 ± 0.14	0.67 ± 0.08	0.90 ± 0.13	0.61 ± 0.03
22:6ω3	7.32 ± 0.86	2.88 ± 0.50*	5.74 ± 0.45	3.99 ± 0.16
Total saturated fatty acids	32.39 ± 1.48	31.46 ± 0.91	26.49 ± 1.52	24.86 ± 0.48
Total monounsaturated fatty acids	22.45 ± 0.70	26.84 ± 0.65	23.40 ± 0.66	27.21 ± 0.75
Total PUFA	45.15 ± 1.61	41.64 ± 0.91	50.12 ± 1.21	47.50 ± 0.82
Total ω6 PUFA	16.88 ± 0.48	23.04 ± 0.44*	16.38 ± 0.42	16.64 ± 0.36
Total ω3 PUFA	28.28 ± 1.15	18.60 ± 0.76*	33.74 ± 1.06	30.84 ± 0.65
ω3/ω6 Ratio	1.67 ± 0.03	0.81 ± 0.03*	2.06 ± 0.08	1.86 ± 0.05

[a] Mean ± SEM of 6 animals; values are weight percent of total fatty acids.
[b] 20:3ω6 and 22:1ω9 were not separable under the conditions used here.
* Significantly different ($p < 0.05$) from marine shrimp.
† Significantly different ($p < 0.05$) from wild crayfish.

Table 2—Fatty acid composition of total lipids from edible portions of wild and pond-reared channel catfish and catfish diet[a]

Fatty acid	Wild catfish	Pond-reared catfish	Catfish diet
16:0	20.63 ± 0.41	19.62 ± 0.68	20.99
16:1ω7	7.48 ± 0.88	4.15 ± 0.30*	4.66
18:0	8.04 ± 1.21	6.67 ± 0.60	9.73
18:1ω9	23.72 ± 3.29	42.45 ± 4.46*	34.05
18:2ω6	2.90 ± 0.60	12.35 ± 0.77*	22.49
20:1ω9	0.83 ± 0.76	0.97 ± 0.09	—
18:3ω3	2.78 ± 0.34	1.66 ± 0.18	2.68
18:4ω3	0.60 ± 0.07	0.96 ± 0.16*	0.55
20:3ω6[b]	0.36 ± 0.06	0.98 ± 0.38*	0.03
20:4ω6	6.82 ± 1.56	1.88 ± 0.55*	0.35
20:4ω3	0.62 ± 0.06	0.16 ± 0.02	0.15
20:5ω3	7.02 ± 0.86	1.49 ± 0.39*	2.17
24:1ω9	0.34 ± 0.18	0.16 ± 0.14	0.16
22:4ω6	0.68 ± 0.08	0.14 ± 0.03*	0.13
22:5ω6	1.34 ± 0.15	0.51 ± 0.15*	0.09
22:5ω3	3.05 ± 0.25	0.98 ± 0.30*	0.36
22:6ω3	13.56 ± 0.84	4.97 ± 1.89*	1.41
Total Saturated fatty acids	28.67 ± 1.44	26.28 ± 1.02	30.72
Total Monounsaturated fatty acids	31.64 ± 4.21	47.64 ± 4.80*	38.87
Total PUFA	39.77 ± 3.21	26.07 ± 3.79*	30.41
Total ω6 PUFA	12.13 ± 1.97	15.85 ± 1.40*	23.09
Total ω3 PUFA	27.64 ± 1.44	10.22 ± 2.49*	7.32
ω3/ω6 Ratio	2.54 ± 0.32	0.62 ± 0.10*	0.32

[a] Mean ± SEM of 6 animals; values are weight percent of total fatty acids.
* Significantly different ($p < 0.05$) from wild catfish.
[b] 20:3ω6 and 22:1ω9 were not separable under the conditions used here.

The data presented above demonstrate that lipids from pond-reared prawn, catfish and crayfish have a fatty acid composition that differs considerably from that of their wild counterparts, especially if they are fed diets in which ω6 PUFA predominate. The greatest difference in the ω3/ω6 ratio was between the pond reared catfish, whose diet consisted primarily of a soy based pellet, and the wild catfish. The smallest difference in the ω3/ω6 ratio was betweened the pond reared crayfish, not fed any additional diet, and the wild crayfish. This implies that the beneficial effects of seafood consumption may not be realized if the seafood is commercially cultured with diets high in ω6 PUFA, resulting in animals with lower ω3 PUFA levels and ω3/ω6 ratios.

While it is known that the lipids of cultured fish will reflect their diet (Ackman, 1974), recently published data on the ω3 content of food (USDA 1986) do not specify the origin of fish and shellfish indicating that the authors may be unaware that differences exist between the lipids of wild and cultured seafood.

However, since the fatty acid composition of fish and shellfish reflect their diet, it may be possible to increase the level of ω3 PUFA in the lipids of cultured seafood by dietary manipulation. Increasing the ω3 fatty acid content of cultured fish diets could result in higher ω3 PUFA levels and ω3/ω6 ratios and may enhance the marketability of aquaculture products. There is also evidence that ω3 fatty acids increase the growth response of cultured fish (Sandifer and Joseph, 1976). The feasibility of increasing the ω3 fatty acid content of the diets of cultured fish and shellfish therefore needs further investigation.

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A Research Note

Tenderization of Meat with Ginger Rhizome Protease

Y. B. LEE, D. J. SEHNERT, and C. R. ASHMORE

ABSTRACT

Beef steaks and sliced beef marinated with different levels of crude ginger extract were evaluated for the tenderness and structural changes. A significant ($P<0.05$) enhancement of tenderness with increasing amounts of ginger extract was observed at low levels of extract (0, 0.05, 0.1, and 0.2 mL/6.5 cm^2 for the steaks; 0, 0.25, 0.5, and 1 mL/100g for the sliced beef), whereas further improvement of tenderness at more than 0.2 mL/6.5 cm^2 or 1 mL/100g was minimal. Electron microscopy of the treated beef revealed the preferential degradation of thin filaments in the I-bands, resulting in extensive fragmentation of myofibrils. Thus, ginger rhizome protease is an effective meat tenderizer and the tenderization is achieved through preferential degradation of thin filaments.

INTRODUCTION

PROTEOLYTIC ENZYMES used in meat cooking have been shown to increase the tenderness of meat as a result of proteolysis of the various meat protein fractions (Miyada and Tappel, 1956; Tappel et al., 1956; Wang et al., 1958a,b; Kang and Rice, 1970). Among the proteolytic enzymes of plant origin, papain, ficin and bromelain have been most extensively studied (Glazer and Smith, 1971) and are most commonly used for tenderization of meat.

More recently, ginger rhizome was investigated as a new source of plant proteolytic enzyme by Japanese researchers (Ichikawa et al., 1973; Ohtsuki et al., 1978; Thompson et al., 1973). The ginger protease is a thiol proteinase with an optimum temperature of 60°C. Rapid denaturation of the enzyme occurs at 70°C. Its proteolytic activity on collagen appeared to be many fold greater than that on actomyosin, and the combined proteolysis of these two muscle proteins resulted in significantly more tender meat (Thompson et al., 1973). In spite of these findings, ginger rhizome is not utilized as a source of effective meat tenderizer but is used primarily as a flavoring for bakery products and sausage seasoning.

The objective of this study is to determine the optimum level of ginger extract for effective tenderization and to elucidate the modifications of myofibrils caused by its proteolytic activity.

MATERIALS & METHODS

FRESH GINGER RHIZOME was purchased from a local supermarket. The rhizome was peeled, sliced, ground in a mortar with pestle, and squeezed through four layers of cheesecloth to produce a crude ginger extract. This crude extract was used as a source of proteolytic enzymes in subsequent application to meat.

Twenty boneless top loin steaks (longissimus), forty bottom round (biceps femoris) and top round (semimembranosus) steaks of 2 cm thickness were fabricated from USDA Good grade carcasses after 7 days of aging at 4°C. Crude ginger extract was applied on the surface of the steaks at levels of 0, 0.05, 0.1, 0.2, and 0.4 mL per 6.5 cm^2. The penetration of the enzyme solution into steaks was facilitated by pressing twice with a block of sixty needles spaced 1 cm apart. The steaks were then held for 1 hr at 4°C, followed by broiling in an oven until the internal temperature, as measured by metal meat thermometer, reached 70°C. After cooling the cooked steaks to 25°C, three core samples of 2 cm diameter were drilled parallel to the muscle fiber from each steak for Warner-Bratzler (WB) shear measurement. In addition, the central portion of each steak was cut and coarsely ground. Twenty grams of ground meat were used for Lee-Kramer (LK) shear measurement as described by Lee (1983). The sensory evaluation of tenderness was also performed by six trained panel members on an 8-point rating scale; 1 = extremely tough, 2 = very tough, 3 = moderately tough, 4 = slightly tough, 5 = slightly tender, 6 = moderately tender, 7 = very tender, 8 = extremely tender.

For the preparation of sliced and marinated beef, frozen sirloin tip roasts (quadriceps femoris) were defrosted overnight and fabricated into thin slices 4 mm thick. The meat slices were randomly divided into several lots which were treated with different levels of ginger extract (0–3 mL per 100g meat) and held for 1 hr at 4°C. The marinated beef was broiled for 7 min to the same degree of doneness (well-done, approximately 78°C) and cooled to 25°C. Lee-Kramer shear press and sensory rating of tenderness were performed as described previously.

A portion of cooked meat was fixed, sectioned and stained as described by Yu and Lee (1986) and electron micrographs were taken to examine the structural changes.

Data were analyzed by analysis of variance and significance between means was determined using Duncan's multiple range test (Steel and Torrie, 1960).

Table 1—Effect of crude ginger extract on tenderness of beef steaks and sliced-marinated beef

Ginger extract levels[a]	WB shear[b] (kg/2 cm core)	LK shear[b] (kg/20g meat)	Sensory[c] tenderness score
Top loin steak (Longissimus)			
0	6.6[d]	107.5[d]	4.9[d]
0.05	6.7[d]	102.2[d]	5.1[de]
0.1	6.2[d]	91.5[e]	5.6[e]
0.2	5.2[e]	79.4[f]	6.6[f]
0.4	4.7[e]	74.3[f]	6.9[f]
Top round steak (Semimembranosus)			
0	6.8[d]	103.5[d]	5.2[d]
0.05	6.3[d]	95.3[d]	5.7[de]
0.1	5.3[e]	85.0[e]	6.1[e]
0.2	4.7[f]	76.2[f]	6.8[f]
0.4	4.6[f]	72.0[f]	7.1[f]
Bottom round steak (Biceps femoris)			
0	9.6[d]	124.0[d]	3.6[d]
0.05	7.5[e]	105.2[e]	4.8[e]
0.1	6.5[f]	97.3[ef]	5.3[ef]
0.2	6.3[f]	92.5[f]	5.7[f]
0.4	6.1[f]	88.2[f]	6.1[f]
Sliced-marinated sirloin tip (Quadriceps femoris)			
0	—	125.2[d]	3.7[d]
0.25	—	113.3[e]	4.5[e]
0.5	—	94.0[f]	6.0[f]
1.0	—	72.6[g]	7.2[g]
2.0	—	70.3[g]	7.1[g]
3.0	—	67.2[g]	7.6[g]

[a] mL/6.5cm^2 for the steaks and mL/100g for the sliced-marinated beef. All the meat samples were held for 1 hr at 4°C after the application of ginger extract.
[b] Means of 4 determinations.
[c] 1 = extremely tough, 2 = very tough, 3 = moderately tough, 4 = slightly tough, 5 = slightly tender, 6 = moderately tender, 7 = very tender, 8 = extremely tender.
[defg] Means with different superscripts in the same column differ significantly ($P<0.05$).

Authors Lee, Sehnert, and Ashmore are with the Dept., of Animal Science, Univ. of California, Davis, CA 95616.

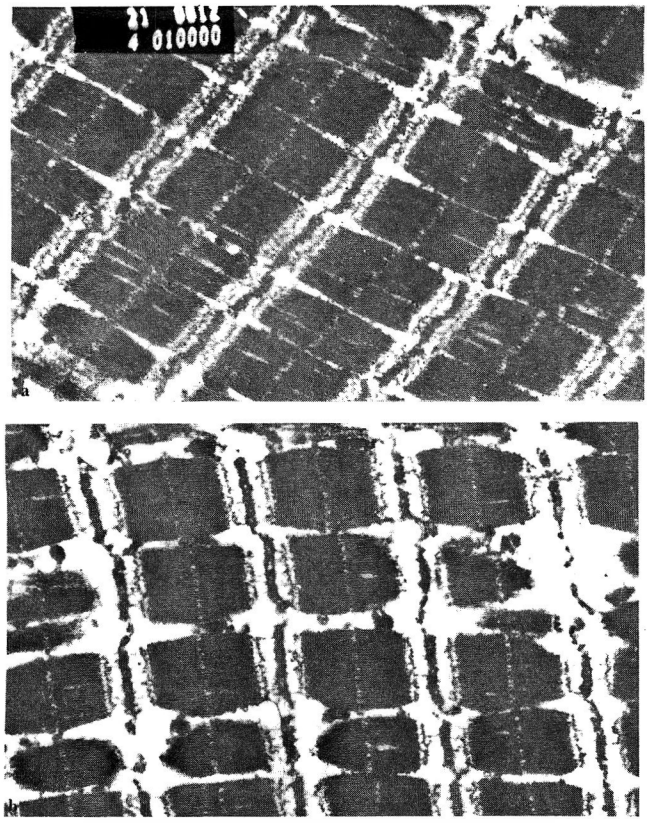

Fig. 1—Electron micrograph of the untreated semimembranous muscle (top) and semimembranous muscle treated with 0.2 mL ginger extract per 6.5cm² surface area (bottom) 27,000×.

RESULTS & DISCUSSION

THE TENDERIZATION effect of crude ginger extract on beef steaks and sliced-marinated beef is summarized in Table 1. For both top loin and top round steaks, an almost linear decrease of shear values with increasing amount of ginger extract was observed between 0 and 0.2 mL per 6.5 cm^2 surface area. A much smaller change in shear values or sensory scores was observed between 0.2 and 0.4 mL. These results indicate that 0.2 mL of crude ginger extract per 6.5 cm^2 surface area appears to be the optimal level to achieve the largest tenderization effect.

A slightly different dose-response was observed in the bottom round steaks. Shear values significantly ($P<0.05$) decreased by the application of 0.05 ml crude extract, followed by a more gradual decline with increasing amounts of extract. The observation of Thompson et al. (1973) that the proteolysis of collagen by the ginger protease was many fold greater than that of actomyosin might explain the sudden decrease of shear value even at very low level of 0.05 mL extract. The untreated bottom round steaks which are known to have higher connective tissue are tougher than either top loin or top round steaks. The inclusion of even a small amount of ginger extract would hydrolyze connective tissue effectively, resulting in greater initial tenderization compared to other steaks low in connective tissue. This hypothesis needs further clarification by more detailed studies.

The tenderization of all the steaks at the levels of ginger extract tested in this study was achieved without a detrimental effect of "mushiness," which has been a problem with other proteolytic enzymes (Rolan et al., 1985).

For the sliced-marinated beef, there also was a linear increase in tenderness at low levels of ginger extract between 0 and 1 mL per 100g meat. The untreated beef was scored slightly tough, whereas the sliced beef treated with 0.5 and 1.0 mL ginger extract was scored moderately tender and very tender, respectively. Further enhancement of tenderness by increasing crude extract above 1 mL per 100g of meat was minimal.

Electron microscopy (Fig. 1) of the treated and cooked meat demonstrates a preferential degradation of thin filaments in the I-bands, resulting in extensive fragmentation of myofibrils. The higher concentration of ginger extract extensively degraded the myofibrils and the degradiation appears to begin at the I-bands of each sarcomere and progress toward the M-line. The low level of ginger extract appears to be sufficient to degrade thin filaments, resulting in fragmentation and enhancement of tenderness. Further increase of ginger extract to more than that necessary for the thin filaments degradation would not enhance the tenderness to a great extent. This explains the earlier observation that the low levels of ginger extract markedly improved the tenderness, whereas further enhancement of tenderness was minimal at higher concentrations.

In conclusion, this study has clearly demonstrated that the ginger protease is an effective meat tenderizer and the tenderization is achieved through the fragmentation of myofibrils by preferential degradation of thin filaments in the I-bands. Further characterization of the proteolytic activity of the ginger protease on connective tissue is in progress.

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A Research Note

Acceptability of Flavor, Texture, and Appearance in Mutton Processed Meat Products Made by Smoking, Curing, Spicing, Adding Starter Cultures and Modifying Fat Source

D. T. BARTHOLOMEW and C. I. OSUALA

ABSTRACT

Mutton meat was tested as the main ingredient, in different processed meat items to obtain prototype products lacking objectionable mutton off-flavors. Four consumer-preference sensory panel sessions were conducted to rate these products against beef or pork controls. Sensory panel results indicated that compared to texture and appearance parameters, flavor had more effect on overall acceptability of these products. Objectionable mutton flavor was apparently reduced by: (1) reducing mutton fat to a level of 10% or less, and (2) using spices, smoking, and/or curing.

INTRODUCTION

THE COMBINED annual per capita consumption of lamb and mutton for 1983 on a retail basis was 0.7 kg (Field et al., 1983) down from 1.95 kg in 1966 (USDA, 1967). Fabricating new lamb/mutton products without mutton off-flavors from primal and nonprimal cuts could help reverse this downward trend.

Lamb and mutton fats contain both species-related flavor components and high levels of saturated fatty acids (Cramer, 1983); mutton flavor also generally increases with age (Field et al., 1983). Researchers agree that meaty aroma comes from the lean portion of meat while species-specific flavors originate in or are deposited in the fat portion (Pearson et al., 1973; Wasserman and Spinelli, 1972).

Reduction of mutton flavor in processed meats has been achieved by reducing mutton fat to a level of 10% or less in processed products (Wenham, 1974; Anderson and Gillet, 1974; Brennand and Mendenhall, 1981), using spices (Baliga and Madaiah, 1971) or smoking and curing (Ogmundsson and Adalsteinsson, 1979).

The purpose of this research was to study the acceptability of mutton processed meat products varying in fat.

MATERIALS & METHODS

THE MUTTON MEAT used in this study came from five Navajo rams. Mutton meat from rams was utilized because of their stronger potential "mutton" flavor, but the results also apply to lamb meat. Deboned meat samples were vacuum packaged and stored in a blast freezer (−30°C) until needed. Beef and pork were obtained locally.

Seven prototype products were made: frankfurters, cured meat rolls, meat rolls, breakfast sausage, meat patties, jerky, and summer sausages using standard processing methods (Rust, 1975; Pearson and Tauber, 1984; Osuala, 1985) (Table 1). Three treatments were evaluated in four consumer-preference sensory panels composed of 67–102 judges: (a) all-beef or all-pork controls, (b) lean mutton (≤10% fat) with or without added beef or pork fat, (c) untrimmed mutton (> 10% fat) with or without added beef or pork fat. The judges evaluated randomized samples for appearance, texture, flavor, and overall acceptance of the products using a hedonic scale from 1 (disliked most) to 9 (liked most) rinsing their mouths with cold water between samples.

The authors are with the Dept. of Nutrition & Food Sciences, Utah State Univ., Logan, UT 84322-8700.

Product processing

Frankfurters were processed using beef (9.5% fat), lean mutton (6.0% fat), untrimmed mutton (16.5% fat), fat pork trim (41.0% fat) or pork fat (87.0% fat) to give meat blocks containing 34% fat.

Cured meat rolls were made with lean mutton (6.0% fat) and pork fat (87.0% fat) to contain less than 10% total fat in the raw formulation. Untrimmed mutton (16.5% fat) and pork fat (87.0% fat) were used to formulate the third cured meat roll containing less than 19% fat. Higher fat levels in uncured mutton treatments were evaluated for acceptability in the cured/uncured meat rolls and jerky because of the increased costs that would be associated with producing trimmed mutton for these products.

Meat rolls were processed using lean mutton or beef to contain less than 10% fat in the raw formulation. Untrimmed mutton (16.5% fat) was used in the third meat roll. Meat patties were made by combining ground lean and fat meats to give 25% fat in the meat blocks. Jerky was made with lean mutton (5.5% fat), lean beef (10.0% fat), or untrimmed mutton (16.5% fat) meat blocks.

Sweet Italian breakfast sausages were formulated to contain 35% fat in the meat blocks using lean mutton (8.0% fat), untrimmed mutton (16.5% fat), pork trim (20.0% fat), and pork fat (87.0% fat). Summer sausages had fat levels of 20% in the meat block using lean beef (10.0% fat), beef fat (87.0% fat), lean mutton (5.5% fat), untrimmed mutton (16.5% fat), or fat mutton trim (45.0% fat).

Preparation of samples for sensory panels

Frankfurters were heated in boiling water for 3 min. Frozen breakfast sausage links were fried for 8 min at 176.6°C. Meat patties were fried at 176.6°C for 4 min on each side. Meat rolls were served warm after heating on a steam table. Cured meat roll, summer sausage, and jerky were served at room temperature.

Fat analysis

Fat was determined by a modified Babcock method (Anonymous, 1971) for adjusting the fat contents of meat blocks in product formulation.

Statistical analysis

Treatment means were compared by Least Significant Difference (LSD) method (Snedecor and Cochran, 1967) for those treatments with significant F-ratios.

RESULTS & DISCUSSION

AN ALL-MUTTON treatment was dropped after the first sensory panel because scores for all-mutton frankfurters were significantly lower than all other treatments ($p \leq 0.05$). The untrimmed mutton treatment had the lowest scores in most of the remaining sensory panel sessions ($p \leq 0.05$; Table 2). Simple linear regressions from sensory panel data were performed to obtain squares of correlation coefficients for flavor, texture, and appearance against overall acceptability (r^2 for

Table 1—Meat product formulations

Ingredients	% of formulation for product no. 1[a]	2	3	4	5	6	7
Lean meat	—	—	—	—	—	94.38	—
Lean meat for bind	—	14.75	16.20	—	—	—	—
2.54 cm cubes - lean meat	—	72.95	82.00	—	—	—	—
Meat block (20% fat)	—	—	—	—	—	—	93.868
Meat block (25% fat)	—	—	—	—	99.00	—	—
Meat block (34% fat)	75.40	—	—	—	—	—	—
Meat block (35% fat)	—	—	—	97.90	—	—	—
Ice	18.80	—	—	—	—	—	—
Cold water	—	9.13	—	—	—	—	—
Seasoning[b]	5.10	—	—	—	—	—	—
Sodium chloride	—	2.00	1.50	1.47	1.00	2.72	2.75
Corn syrup solids	—	—	—	—	—	—	1.50
Sucrose	—	0.90	—	—	—	1.89	1.00
Liquid smoke	0.50	0.203	—	—	—	0.50	0.20
Sodium tripolyphosphate	—	—	0.30	—	—	—	—
Ground black pepper	—	—	—	—	—	0.32	0.23
Ground white pepper	—	—	—	0.25	—	—	0.17
Garlic powder	—	—	—	—	—	0.013	0.015
Monosodium glutamate	—	—	—	—	—	0.11	0.20
Sodium erythorbate	—	0.055	—	—	—	0.055	0.055
Sodium nitrite	—	0.012	—	—	—	0.012	0.012
Prague powder	0.20	—	—	—	—	—	—
Whole fennel seed	—	—	—	0.25	—	—	—
Paprika	—	—	—	0.13	—	—	—
	100.00	100.00	100.00	100.00	100.00	100.00	100.00

[a] 1 = frankfurters, 2 = cured meat rolls, 3 = meat rolls, 4 = sweet Italian breakfast sausages, 5 = meat patties, 6 = jerky, 7 = summer sausages.
[b] Frank seasoning 026-0033, Griffith Labs, Inc., Chicago, IL.

Table 2—Means of sensory panel parameters (flavor and overall acceptability) for meat products

Products	Treatments	Flavor[a]	Overall acceptability[a]
Frankfurters	Beef/fat pork trim	6.124[b]	6.062[b]
(97 judges)	Lean mutton/pork fat	6.320[b]	6.206[b]
	Untrimmed mutton/pork fat	5.474[c]	5.557[c]
	LSD*	0.514	0.475
Cured meat rolls	All-pork	6.736[b]	6.486[b]
(72 judges)	Lean mutton/pork fat	6.000[c]	5.903[c]
	Untrimmed mutton/pork fat	6.139[c]	6.125[bc]
	LSD*	0.577	0.562
Meat rolls	Lean beef	5.562[b]	5.192[c]
(73 judges)	Lean mutton	5.603[b]	5.329[c]
	Untrimmed mutton	5.288[b]	5.137[c]
	LSD*	0.635	0.628
Breakfast sausages	All-pork	5.075[b]	4.925[b]
(67 judges)	Lean mutton/pork fat	4.925[b]	4.910[b]
	Untrimmed mutton/pork fat	5.015[b]	4.925[b]
	LSD*	0.669	0.609
Meat patties	All-beef	7.130[b]	7.000[b]
(69 judges)	Lean mutton/beef fat	5.638[c]	5.681[c]
	Untrimmed mutton/beef fat	5.000[d]	5.116[d]
	LSD*	0.592	0.502
Jerky	Lean beef	6.990[b]	6.667[c]
(102 judges)	Lean mutton	6.863[b]	6.627[c]
	Untrimmed mutton	6.392[c]	6.255[c]
	LSD*	0.457	0.469
Summer sausages	All-beef	6.960[b]	6.931[b]
	Untrimmed mutton/beef fat	6.525[c]	6.535[bc]
	LSD*	0.431	0.410

[a] Sensory panel parameter was rated on a hedonic scale of 1–9 (1 = disliked the most and 9 = liked the most). Means within a product in the same column with the same letter are not significantly different ($p = 0.05$).
* Least significant difference ($p = 0.05$).

frankfurters of 0.741, 0.491, 0.272, respectively). Flavor had the greatest effect on overall acceptability of each meat product; therefore, only flavor and overall acceptability are reported.

Fat modification effects

Objectionable mutton flavor was apparently reduced by trimming all visible fat from the meat so the level was less than or equal to 10% due to improved product flavor. Lean portions were then combined with beef or pork fat. Elimination of mutton flavor by fat removal agrees with previous work (Wenham, 1974; Anderson and Gillet, 1974; Brennand and Mendenhall, 1981).

Sensory panelists liked the flavor better when lean mutton was included in meat patties, frankfurters or jerky ($p \leq 0.05$, Table 2). Jerky and frankfurters containing trimmed mutton were equal to the all-beef products in flavor acceptability ($p \leq 0.05$).

Spice effects

Mutton products containing higher levels of spice appeared to be more acceptable. Products in descending order of non-meat ingredient levels (%) including spices and hot water, and flavor scores for the untrimmed mutton treatment (Tables 1 and 2) were: summer sausage (6.13%, 6.52), frankfurter (5.80%, 5.47), jerky (5.61%, 6.39), cured meat roll (3.11%, 6.13), breakfast sausage (2.10%, 5.01), meat roll (1.80%, 5.28), and meat patty (1.00%, 5.00). Effectiveness of spices masking objectionable mutton flavor is in agreement with the work of Baliga and Madaiah (1971). More work is needed to determine which spices and spice combinations make the best masking agents.

Product evaluation

Scores of 6 and above assigned to products by the sensory panel suggest that mutton summer sausages, jerky, cured meat rolls and frankfurters might be commercially acceptable.

CONCLUSIONS

PRODUCT ACCEPTABILITY was increased and objectionable mutton flavor was apparently reduced by trimming mutton fat to 10% or less and substituting beef or pork fat for mutton fat. Ratings of product acceptability by consumer preference sensory panels indicated that mutton had the potential for commercial use as the predominant meat ingredient in processed meat products, especially summer sausages, frankfurters, cured meat roll, and jerky.

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A Research Note

Thiamin, Riboflavin, and Nicotinic Acid Contents of Tropical Root Crops from the South Pacific

J. H. BRADBURY and U. SINGH

ABSTRACT

The thiamin, riboflavin, and nicotinic acid contents of sweet potato (*Ipomea batatas*, taro (*Colocasia esculenta*), giant taro (*Alocasia macrorrhiza*), giant swamp taro (*Cyrtosperma chamissonis*), taro (*Xanthosoma spp*), yam (*Dioscorea alata* and *D. esculenta*) were determined for fresh and 40°C dried material obtained from six South Pacific countries. Losses on drying at 40°C for 2–3 days were 10–15% for the three vitamins. Sweet potato contained the largest amount of thiamin (40–120 μg/100g fresh weight) and along with *Colocasia esculenta* and *Xanthosoma spp*. the largest amounts of nicotinic acid. The root crops provided inadequate amounts of thiamin, riboflavin, and nicotinic acid with values ranging from 12–123, 12–59 and 220–1310 μg/100g fresh weight, respectively. Losses on cooking were about the same for all vitamins and root crops, with about a 20% loss on boiling (water retained) or baking and about a 40% loss on boiling (water discarded).

INTRODUCTION

THE LEVELS of total vitamin C in tropical root crops were previously determined by Bradbury and Singh (1986). The thiamin, riboflavin and nicotinic acid of foods have been determined including losses during storage and processing (Dwivedi and Arnold, 1973; Girija et al, 1982; Okoh, 1984; Gregory, 1984). The objective of this study was to determine the thiamin, riboflavin and nicotinic acid of cooked and uncooked tropical root crops from the South Pacific.

MATERIALS & METHODS

Materials

Papain (type II), α-amylase, (Type II), and phosphatase (type II) were obtained from Sigma Chem. Co. (St. Louis, Mo). Thiamin, riboflavin, nicotinic acid, cyanogen bromide, and other chemicals were reagent grade. Freshly harvested and weighed roots or stems were air freighted from the South Pacific and stored at 15°C for a short time before processing. Roots or stems were peeled, chopped and dried at 40°C to constant weight to determine moisture loss (Bradbury et al., 1984, 1985). Duplicate analyses for vitamins were made on the dried samples. Fresh sweet potato and giant taro samples were peeled and divided into three parts, proximal, middle, and distal. From each part, two samples were taken from the periphery (2–3 mm beneath the skin) and a third sample from the center. These samples were analyzed and moisture determined at 40°C.

For cooking ~50g cubes of root crop were heated in boiling water for 10, 20, and 30 min. In one case the cooking water was discarded and in another it was retained and evaporated to dryness in the presence of the boiled sample which was then dried at 40°C. Baking of ~50g cubes was at 200°C in an oven for 15, 30, and 45 min. Samples were dried at 40°C to constant weight and analyzed. A control sample was processed as above but without boiling and baking.

Author Bradbury is with the Chemistry Dept., Australian National Univ., Canberra, A.C.T., 2601, Canberra. Author Singh is Visiting Fellow at Australian National University on sabbatical leave from International Crop Research Institute for Semi Arid Tropics (ICRISAT). Current address: ICRISAT, Patancheru 502324, AP, India.

Methods

Thiamin. Fresh (about 4g) or dry (about 1.5g) material was homogenized in 40 mL of 0.1M HCl for 3 min using a Polytron (Kinematica, GMBH, Switzerland). The mixture was heated for 1 hr at 100°C, cooled, and made to 50 mL with 0.1M HCl. After centrifugation at 10,000 *g* for 10 min, 10 mL supernatant was used for analysis. The AOAC (1980) method was used for oxidation. The solution was extracted with 20 mL isobutanol and its fluorescence was measured in a Perkin Elmer Model 512 fluorescence spectrometer in the excitation mode with excitation wavelength 370 nm and emission wave length 445 nm.

In order to study the affect of enzyme extraction on thiamin estimation, the 0.1M HCl extract after heat treatment was adjusted to pH 4.3 with 2M sodium acetate. An enzyme solution (5 mL) containing 100 mg papain, 50 mg α-amylase and 50 mg phosphatase in 100 mL 1M sodium acetate buffer (pH 4.3) was added and incubated for 3 hr at 38°C. The mixture was heated at 100°C for 15 min to inactivate the enzymes and was processed as above. The enzyme blank was prepared in a similar way. Extraction in 1M sodium acetate (pH 4.3) for 16 hr at 38°C instead of 0.1 M HCl was also studied in absence and presence of enzymic digestion.

Riboflavin. About 4g fresh or 1.5g dry material was homogenized in 40 mL of 1M sodium acetate buffer (pH 4.3) for 3 min. The homogenate was heated for 1 hr at 100°C, cooled and made to 50 mL with distilled water. After centrifugation at 10,000 *g* for 10 min, the clear supernatant was collected and 10 mL was used for analysis (AOAC, 1980). The fluorescence was measured with excitation wavelength 440 nm and emission wavelength 530 nm.

Nicotinic acid. Fresh sample (about 5g) or dry powder (about 2.5g) was homogenized in 40 mL of 0.5M H_2SO_4 for 3 min. The mixture was refluxed for 1 hr, cooled and pH adjusted to 4.5 with 10M NaOH. The AOAC (1980) colorimetric method was followed.

RESULTS & DISCUSSION

FOUR DIFFERENT PROCEDURES were compared for extraction of thiamin. Extraction of sweet potato and yam by acetate buffer (pH 4.3) alone gave 25–37% lower results than extraction with 0.1M HCl. Addition of enzymes increased the degree of extraction of sweet potato, but results were 10% lower than using 0.1M HCl. Additional treatment with enzymes after the 0.1M HCl extraction, caused no increase in recovery of thiamin. With taro and giant taro, extraction was maximal in all cases. Thus, the extraction procedure with boiling 0.1M HCl (no enzymes) was found satisfactory in all cases in confirmation of Gubler (1984).

Analysis of fresh samples of sweet potato, taro, and giant taro and the same samples after drying to constant weight at 40°C showed losses of about 15% of thiamin, 12% of riboflavin and 10% of nicotinic acid in the samples dried at 40°C. These losses, which were outside experimental error, may have been due to decomposition on drying for 3 days at 40°C, or perhaps due to inability to extract fully the vitamins from dried powder. Sun drying of Nigerian vegetables caused losses of thiamin and riboflavin, due to photochemical and heat degradation (Okoh, 1984).

Riboflavin was found to be constant across a sweet potato root and giant taro stem. Thiamin of sweet potato was twice as large 2–3 mm below the skin compared with the center and for giant taro it was 30% higher near the skin than at the center.

Table 1—Thiamin (T), riboflavin (R), and nicotinic acid (N) content of tropical root crops from various South Pacific countries[a]

Crops	No. of cultivars per entry	Vitamin analysed	Vitamin content from South Pacific countries: Papua New Guinea	Solomon Islands[a]	Tonga	Fiji	Western Samoa	Kiribati	Total range of values
Sweet potato	3	T	85(30)	73(20)	99(18)				43- 123
		R	25(2)	41(13)	27(6)				19- 59
		N	770(96)	656(121)	384(94)				259- 887
Taro	4	T		37(22)		35(13)	25(6)		15- 71
(*C. esculenta*)		R		17(6)		34(7)	25(6)		16- 40
		N		678(368)		932(268)	671(205)		268-1310
Giant taro	4	T					23(6)		15- 32
Alocasia		R					20(6)		12- 29
macrorrihiza		N					531(203)		220- 769
Giant swamp taro	4	T						25(19)	12- 59
Cyrtosperma		R						19(5)	12- 26
chamissonis		N						463(112)	385- 644
Taro	3	T			24(7)				14- 29
Xanthosoma spp.		R			28(8)				25- 36
		N			798(202)				711-1078
Yam	5	T	31(7)	63(24)					23- 90
D. alata		R	24(7)	36(10)					15- 53
		N	335(77)	408(72)					245- 490
Yam	5	T	45(17)	44(11)					24- 72
D. esculenta		R	26(3)	30(10)					18- 44
		N	378(150)	450(147)					251- 691

[a] Mean values (standard deviation in brackets) in μg/100g fresh weight. Cultivars of root crops used are as follows: *Sweet potato* (PNG) K-9, WMN, KO-2, (Sol. Is.) reef jimi, bugotu, dingale, (Tonga) tongamai, hawaii, halasika; *Taro* (Sol. Is.) PD-41, sasagiha, PD-1, PD-12, (Fiji) samoa normal, samoa hybrid, toakula, tausala ni samoa, (W. Samoa) niue, manua, fae'k'ele, pae'pae' *Giant taro* (W. Samoa) sega, toga, fui, niukini; *Giant swamp taro* (Kiribati) katuta red, ikarao red, ikarao green, atimainiku; *Taro Xanthosoma spp.* (Tonga) futuna, maheleuli, tea; *Yam D. alata* (PNG) takua yaimbi, du kupmi, yavovi, tolai, kpmora, (Sol. Is.) UL-5, toki, WCH-9, GU-147, A-172; *Yam D. esculenta* (PNG) mangilmu, glame, saikidi, kualika, martka, (Sol. Is.) fananiu, NGP-3, GUP-11, GUP-5, GUP-7.

There was no gradient of thiamin concentration from proximal to distal end in sweet potato or giant taro. No measurements were made for nicotinic acid.

The results in Table 1 are averages of analyses of 3–5 cultivars of a root crop from a particular country. Close examination of the data for each cultivar (not given in Table 1) failed to show any particular cultivars which contained consistently high levels of all three vitamins compared with others. In Table 1, the thiamin content of sweet potato was significantly greater ($P<0.01$, t-test) than that of the other root crops. The riboflavin content was not significantly different from one root crop to another, but taro (*C. esculenta*) from Fiji contained significantly more riboflavin ($P<0.05$) than taro from Solomon Islands. Nicotinic acid was variable and was higher in taro and sweet potato ($P<0.05$) than in yam.

The values in Table 1 were made more meaningful by calculation of the amount of root crop that would be needed to supply the recommended daily allowance of thiamin (1.4 mg), riboflavin (1.6 mg) and nicotinic acid (19 mg) (Davidson et al., 1979). The amount of fresh root crop required was within the range 1.6–8.4 kg/day. The adequacy of riboflavin was less than for thiamin and nicotinic acid in virtually all cases. Clearly, intake of these vitamins would need to be augmented from other sources.

The breakdown of vitamins on cooking increased with increase in the time of boiling or baking and the loss was independent of the type of root crop and of the vitamin measured. The loss of thiamin, riboflavin, and nicotinic acid averaged 20% (standard deviation, SD 6%) after boiling for 20 min (water retained), 39% (SD 9%) (water removed) and 23% (SD 6%) after baking for 30 min. Samples were edible after 20 min boiling and 30 min baking. The approximate doubling of the vitamin loss on boiling if the water were discarded, was due to extraction of water soluble vitamins, as previously found with total vitamin C (Bradbury and Singh, 1986). The losses of the three vitamins are of the order of 20% on baking or on boiling (water retained) or 40% on boiling (water discarded) and may be compared with other heat treatment studies of these vitamins in sweet potato (Junek and Sistrunk, 1978), rice (Smirnova et al., 1982), legumes (Kilgore and Sistrunk, 1981; Soetrisno et al., 1982) and vegetables (Okoh, 1984).

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A Research Note

Use of Sensory Analysis for the Observation of Single Gene Effects on the Quality of Canned Peas (*Pisum sativum* L.)

G. R. TAURICK and M. R. MCLELLAN

ABSTRACT

Six genetic lines of peas isogenic for the A locus were used in a study of canned pea quality. This gene controls the expression of pigmentation on several parts of the pea plant and seed and is known to influence the quality of fresh and processed peas. Specific sensory attributes investigated were greenness, grayness, liquor clarity, typical pea aroma, grassy (hay-like) flavor, firmness, mushiness, skin toughness, and acceptability level. Alleles of the A locus were shown to control appearance, flavor and general acceptability of canned peas but were not associated with textural differences. Use of formal methods in sensory analysis to characterize specific genetic effects may be a powerful tool in the identification of genetic control centers for quality characteristics.

INTRODUCTION

ALL GREEN PEA (*Pisum sativum* L.) cultivars currently in use in the United States for processing, fresh use or dry peas are homozygous recessive at the *aa* locus. Pea plants carrying a dominant *A* allele have the capability to produce flowers, axils, internodes, pods and seed coats, while plants homozygous recessive for *aa* are uniformly green with white flowers and white or pale green seed coats. Dominance at *A* is associated with favorable agronomic characteristics, notably resistance to seedling and root-rot diseases (Ewing, 1959; Kraft, 1977; Stasz et al., 1980), but pea breeders select against dominant *A* alleles because of negative effects on quality, such as dark color, off-flavor and cloudy can liquor. Peas with dominant *A* are said to have acrid (Hedrick, 1928), astringent taste (Ewing, 1959), or off-flavors (Muehlbauer and Kraft, 1978) and objectionable color (Muehlbauer and Kraft, 1978). Some edible-podded pea varieties, however, are dominant at *A* (e.g., Mammoth Gray Pod). Edible-podded peas are consumed in an immature state, before negative quality aspects of the dominant *A* allele are expressed. The current research has identified some of the specific effects of the *A* locus on quality parameters of canned peas.

Sensory panels have previously been used to evaluate quality of canned peas, but generally only to compare factors such as cultivars, processing methods, cultivation, harvesting or post-harvest conditions. For example, McCurdy et al. (1983) used taste panels and chemical and physical determinations to compare effects of cultivar, soak solution, blanch method and brine composition on canned quality of dry pea cultivars 'Alaska' and 'Garfield'. The application of formal sensory methods to the characterization of single gene effects has not been previously reported.

In this study, effects of dominance or recessivity of gene *A* on specific sensory quality components were observed in six genetic lines of peas isogenic for the *A* locus.

Author Taurick is affiliated with the Dept. of Horticultural Sciences and author McLellan is with the Dept. of Food Science & Technology, New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456.

MATERIALS & METHODS

SIX LINES OF PEAS with different genetic backgrounds were self-pollinated for 8 to 11 generations, selecting for heterozygosity at the *A* locus at each generation. These lines can be considered to be essentially homozygous at all other loci, and are, therefore, nearisogenic (identical at all other genes). Any differences within isolines can therefore be attributed to effects of the segregating gene.

Plants were grown at the Vegetable Research Farm of the New York State Agricultural Experiment Station in small field plots in 1984 and 1985 and harvested by hand. Peas were removed from pods and vines in a stationary viner; tenderometer values were determined for each lot. After size-grading, peas were blanched for 3 min in boiling water and packed into 303 size cans (325 g/can). Cans were filled with boiling distilled water (1984 season) or 2% NaCl brine (1985 season) and processed in an FMC Steritort for 6.2 min at 260°F (126.6°C).

The 1984 evaluation was done by a preference panel. Thirty untrained panelists were randomly selected from the staff of these departments. Each was presented sequentially with five pairs of pea samples identified by 3-digit random numbers. Each pair consisted of one *AA* and one *aa* sample of one line; each panelist tasted one pair from every line. The order of these paired samples was random in presentation. Samples (~20g) were put into small plastic souffle dishes and preheated in a microwave oven before presentation. Presentation was done in our taste panel facilities under controlled ambient temperature and lighting conditions. Panelists were asked which sample from each pair they preferred, and to note what characteristics were important in making their choices.

The 1985 panel was run using quantitative descriptive analysis (QDA). A modified open discussion panel (McLellan et al., 1984) was held prior to the QDA panel in 1985 to decide which descriptors to include on the ballot for the QDA panel. Six experienced panelists were presented with four preheated pairs of samples. The panel discussed visual, odor, taste and textural characteristics that could be used to describe the range of differences present between *AA* and *aa* samples. From this discussion a sensory ballot listing specific descriptors was derived. The descriptors were: greenness (light green – dark green); grayness (light gray – dark gray); liquor (cloudy – clear); typical pea aroma (weak – strong); grassy (hay-like) flavor (weak – strong); firmness (firm – tender); mushiness (not mushy – mushy); skin toughness (soft – chewy); and acceptability level (low – high).

The QDA panel consisted of eight trained members. Each of these panelists had undergone training in the technique of QDA methods and each had previous panel experience with the method. Most of the eight were on the open discussion panel and as such were well acquainted with the descriptors being used. Those who were not on that previous panel were thoroughly briefed on the descriptors being utilized. Each of six lines was represented by two alleles (homozygous dominant or recessive at the *A* locus) and three replicates per allele, so that there were 36 samples for each panelist. All 36 samples were presented in random order, six at each of six sittings. The samples were identified by three-digit random numbers and preheated as before. The panelists were asked to make a mark on the ballot for each descriptor, agreeing with the degree that they felt the descriptor was expressed in each sample. The ballots were later digitized, so that each mark was given a value from 0 – 10, and effects of lines and alleles were compared using analysis of variance.

RESULTS & DISCUSSION

1984 Preference tests

Results from the 1984 preference test are shown in Table 1. In every case more panelists preferred the sample recessive for

Table 1—Results of the 1984 preference test for canned peas. Thirty panelists evaluated paired samples (one AA and one aa) from each of six lines of peas and indicated which sample was preferred in each case

Line #:	3		4		5		8		9	
Allele	*AA*	*aa*	*AA*	*aa*	*AA*	*aa*	*AA*	*aa*	*AA*	*aa*
# Preferred	10	20	5	25	3	27	1	29	11	19
Significance	*		**		**		**		-NS-	

* 95% Significance level
** 99% Significance level
NS Not statistically significant

Table 2—Results of analysis of variance of 1985 Quantitative Descriptive nalysis panel for canned peas. (Eight panelists sampled six lines X two alleles X three replicates/allele = 36 samples, presented in random sequence

Attribute	Range	Mean[a] AA	Mean[a] aa	Allele F Value
Acceptability	(low-high)	2.09	4.29	140.42**
Greenness	(light-dark)	3.49	2.73	19.37**
Grayness	(light-dark)	5.33	0.93	631.21**
Cloudy Liquor	(cloudy-clear)	3.92	4.92	37.551**
Grassiness	(weak-strong)	4.19	3.04	23.06**
Aroma intensity	(weak-strong)	2.80	3.73	17.30**
Firmness	(firm-tender)	4.90	5.18	1.40 NS
Mushiness	(not mushy-mushy)	5.05	4.71	2.05 NS
Skin Toughness	(soft-chewy)	6.00	5.61	2.36 NS

* 95% Significance level
** 99% Significance level
NS Not statistically significant
[a] Responses were given values from 0–10, where 0 represents the left-hand range characteristic, and 10 represents the right-hand range characteristic. The means of all panelist responses for each attribute are given.

aa; the difference was significant or highly significant for four out of the five lines. Descriptors listed by the panelists included 108 flavor terms such as sweet, bitter, bland, starchy, pea flavor; 69 color terms such as dark, green, gray, bad color; and 50 textural terms such as mealy, firm, mushy.

1985 Quantitative descriptive analysis

Differences between genetic lines were highly significant for all components except "acceptability". Line differences were expected; these were experimental lines that have not been subjected to selection for normal horticultural characteristics and were different from one another in many ways. There was no significant difference between the lines for acceptability. Of specific interest in this study were the effects due to allele (Table 2). The *A* locus was highly correlated with acceptability; recessive *aa* was always preferred. Allele effects were highly significant for the three appearance characteristics; *AA* samples were darker gray, darker green, and had cloudier liquor. The descriptor phrase "dark green" probably confounded two different appearance components, darkness and greenness. The *AA* samples were darker but the color quality was more of a brown or reddish-brown than green. The "greenness" statement might have better been described as a range from "not green" to "very green". The flavor characteristics were both highly significant. Dominance at *A* caused stronger grassy flavor and weaker typical pea aroma. There were no significant differences due to allele in any of the three textural characters, firmness, mushiness or skin toughness.

CONCLUSIONS

A QDA TASTE PANEL, using trained panelists, found that dominance of the *A* gene in canned peas caused the peas to have darker color and cloudier liquor, with stronger grassy flavor and weaker typical pea aroma. Allelic state of the *A* locus did not produce noticable difference in texture. Use of isogenic lines enabled effects of a single gene to be determined. Quantitative descriptive analysis was found to be a useful tool for measuring the effects of the *A* gene on quality of canned peas. The authors suggest that this approach in genetic study could lead to a greater understanding of genetic effects on overall quality of many commodities.

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A Research Note

High Performance Liquid Chromatographic Determination of Vitamin C in Fresh Tomatoes

L. F. RUSSELL

ABSTRACT

A reverse phase ion-pairing HPLC method for determining vitamin C in fresh tomatoes was developed. An internal standard was used for calibration and quantitation. Recoveries of vitamin C from fresh tomato samples that had been spiked with known amounts of the acid ranged from 96 to 105%. Determination of the vitamin C in fresh tomatoes by this method compared favorably with similar analyses using the standard indophenol titration method.

INTRODUCTION

THE WIDELY USED dichloroindophenol dye titration (AOAC, 1984), based on the ability of vitamin C (L-ascorbic acid, AA) to reduce the dye, suffers from two principal disadvantages: (1) foods, including fresh fruit and vegetables, contain interfering substances in addition to AA which can reduce the dye, and (2) the endpoint is not well-defined, especially in pigmented extracts (Sood et al., 1976; Watada, 1982). In an effort to overcome these problems, a number of HPLC methods for the analysis of vitamin C in foods have recently been published. These methods can be divided into four major groups: (1) ion exchange chromatography (Floridi et al., 1982); (2) separations using bonded amino functional groups (Haddad and Lau, 1984); (3) reverse phase chromatography (Speek et al., 1984); and (4) reverse phase ion pair chromatography (Keating and Haddad, 1982).

In the majority of the published HPLC methods, AA was eluted very close to the void volume, making them unsuitable for use in the present investigation. The advantages of using internal standards to compensate for errors such as variations in the injection volume or losses during sample preparation are well recognized (Snyder and Kirkland, 1979). Of the recent HPLC procedures for vitamin C, only two ion exchange methods (Rizzolo et al., 1984; Vanderslice and Higgs, 1984) and one amino bonded chromatography methods (Doner and Hicks, 1981) made use of internal standards. However, rapid, irreversible "poisoning" of ion exchange and bonded phase columns by artifacts in the extracts has been reported by several workers (Sood et al., 1976; Moledina and Flink, 1982; Haddad and Lau, 1984) and was observed during the preliminarly stages of the present study. The objective of this investigation was to develop a high performance liquid chromatography (HPLC) method, including internal standard, for the determination of vitamin C in fresh tomatoes.

MATERIALS & METHODS

Sample preparation

Samples of four to six table-ripe tomatoes were purchased locally on the day before analysis and stored at room temperature overnight. A 100-g sample and a 100-g aliquot of 6% metaphosphoric acid solution were blended for 60 sec at 2650 rpm using a Polytron homogenizer (Brinkmann). Weights instead of volumes were used to overcome errors introduced by foam formation during blending (Russell et al., 1983). A 50-g aliquot of this slurry was mixed with 35 mL of methanol and 10 mL of the internal standard, 0.05% pentanophenone (Pierce Chemical Co., Rockford, IL) in methanol. The mixture was centrifuged for 15 min at 13,300 × *g* at 4°C. The supernatant was decanted and diluted to 100 mL with 50% methanol. A 10-mL aliquot of this solution was further diluted to 100 ml with 1.5 mM pyrogallol in 50% methanol; a portion was passed through a 0.45 μm filter before injection into the HPLC.

Author Russell is with the Food Research Centre, Agriculture Canada, Ottawa, Ontario, Canada K1A OC6.

Calibration curves

A series of standard solutions containing 0.6% metaphosphoric acid, 0.0005% pentanophenone, 1.3 mM pyrogallol and 2.5 to 12.5 μg/mL AA in 50% methanol was prepared immediately before analysis. The ratio of the peak areas for AA to the internal standard was plotted against the concentration of AA. The equation for this calibration curve was determined by linear regression analysis. Analytical results were expressed as mg AA/100g fresh weight.

Chromatographic conditions

HPLC analysis was conducted using a Perkin-Elmer Series 4 liquid chromatograph with column oven set at 35°C and a Perkin-Elmer LC-75 detector set at 247 nm, sensitivity 0.04 AUFS. Data were collected on a Hewlett-Packard 3390A recording integrator. The analytical column was a 25 cm × 4.6 mm Vydac 201 HS column, 10μ particle size (The Separations Group, Hesperia, CA) and was preceded by an RP-18 guard column (Brownlee Labs, Santa Clara, CA). The flow rate was set at 3.5 mL/min. The mobile phase was 0.5 mM tridecylammonium formate in 60+40+1 methanol + water + acetonitrile, pH 4.25. It was prepared by modifying the method of Augustin et al. (1981) as follows: (1) the pH was adjusted to 4.25 using formic acid, and (2) 10 mL of acetonitrile were added per L of solution to reduce peak tailing. All solvents were HPLC grade. Water was deionized and distilled in glass. Before use, the mobile phase was passed through a 0.45 μm filter.

To preserve separation efficiency and prolong column life, the following HPLC maintenance procedures were found to be essential. At the end of every day, the HPLC system was flushed with 30 column volumes of methanol, followed by 1 + 1 methylene chloride + methanol, and methanol again. Approximately once every 2 weeks a flow program of 20 column volumes each of acetonitrile, acetone, methylene chloride, hexane, methylene chloride, acetone, and acetonitrile was used to wash the column thoroughly. When the HPLC system was not operating, the column contained methanol (Augstin et al., 1981; Snyder and Kirkland, 1979).

Titrimetric determination of Vitamin C

Vitamin C was determined by the dichloroindophenol dye titration method (AOAC, 1984) as modified by Russell et al. (1983). Results were expressed as mg AA/100g fresh weight.

RESULTS & DISCUSSION

THE HPLC METHOD, as developed, was used to obtain the chromatogram of a fresh tomato extract, (Fig. 1). Metaphosphoric acid, pyrogallol and artifacts extracted from the tomatoes were eluted at or near the void volume. Both the AA and pentanophenone peaks were baseline resolved and the separation between AA and the unretained compounds was adequate; analysis time was 7.50 min.

The analytical problems associated with quantitive extraction of a nutrient as labile as vitamin C are well documented (Association of Vitamin Chemists, 1966). Attempts to chromatograph samples of AA in 6% metaphosphoric acid solution yielded long, broad metaphosphoric acid peaks that tailed badly and masked the AA. To reduce the concentration of metaphosphoric acid enough to permit the baseline resolution of the AA peak but continue to protect the AA from oxidation, an antioxidant solution containing 1.5 mM pyrogallol was used for the final dilution of extracts. AA in solution is known to

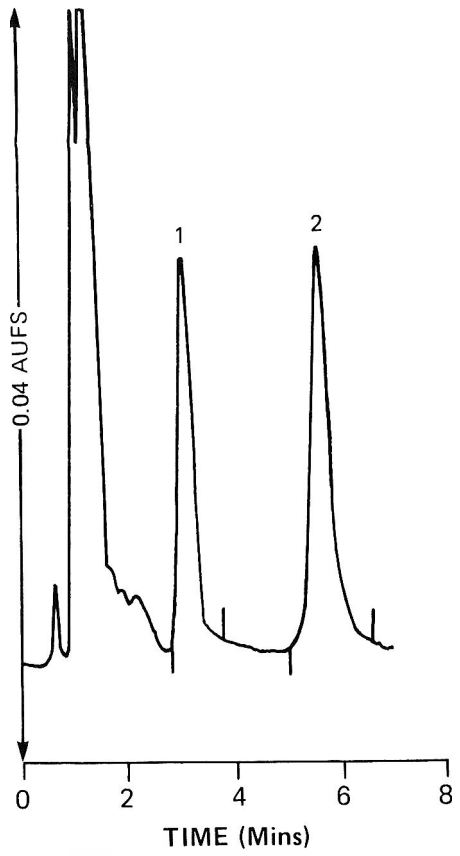

Fig. 1—Chromatography of table-ripe fresh tomatoes: (1) Vitamin C, retention time = 3.02 min.; (2) the internal standard, pentanophenone, retention time = 5.58 min.

be unstable at pH values above 4.2 (AOAC, 1984). In the interests of increased AA stability, it was found that the pH of the mobile phase of Augustin et al. (1981) could be reduced from 4.5 to 4.25 without deleterious effects on the chromatography.

Pentanophenone proved to be the most suitable of the compounds evaluated for use as the internal standard. Using the chromatography conditions established for optimal AA separation, pentanophenone was well separated from AA without lengthening analysis time unacceptably and it did not co-elute with artifacts in the tomato extracts. The extraction procedure was modified to include methanol, thus permitting the early addition of the internal standard to compensate for losses during sample preparation.

Detector response was linear over the range 2.5 to 12.5 μg AA/mL. Because of the excellent linearity of the experimental data for standards, it can therefore be concluded that the use of a single-point standard is justified. The lower limit for the quantitation of AA by this method was comparable to limits of detection ranging from 20 to 50 ng reported in the literature, (Geigert et al., 1981; Doner and Hicks, 1981; Wimalasiri and Wills, 1983; Vanderslice and Higgs, 1984). Tomato samples were spiked with known quantities of AA to test the efficiency of the HPLC method. Recoveries ranged from 96 to 105%.

The AA content of six samples of fresh tomatoes was determined by both HPLC and the commonly used dichloroindophenol dye titration method (Table 1). Agreement between the two methods was satisfactory. For five of the samples, the HPLC and titration results differed by 2% or less. The variation

Table 1—Comparison of Vitamin C of fresh tomatoes determined by HPLC and by dichloroindophenol titration

	Vitamin C (mg/100g fresh weight)	
Sample	By HPLC	By Titration
1	18.8 ± 0.8 (6)[a]	18.6 ± 1.0 (6)
2	13.1 ± 0.4 (6)[a]	12.9 ± 0.6 (6)
3	17.6 ± 1.0 (6)[a]	17.6 ± 0.9 (6)
4	14.9 ± 0.6 (6)[a]	14.6 ± 0.3 (6)
5	15.9 ± 0.7 (5)[a]	15.8 ± 0.9 (6)
6	17.3 ± 0.8 (5)[a]	16.6 ± 0.5 (6)

[a] (N) = number of determinations

for the sixth sample was 4%. The variability of the two methods, as represented by the standard deviations, was similar.

In contrast to many of the other HPLC procedures, this method offers increased flexibility because the retention of AA on the column is such that the method is applicable to extracts that contain artifacts eluting at the void volume. In addition, incorporation of an internal standard into this method provides built-in verification of analytical accuracy that is not available in the majority of the published methods.

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A Research Note

Extraction of Milk Clotting Enzyme from Sodom Apple (*Calotropis procera*)

OGUGUA C. AWORH and S. NAKAI

ABSTRACT

A milk clotting enzyme with low proteolytic activity was extracted with ammonium sulfate, at 0.40–0.65 saturation, from sodom apple leaves. The enzyme with apparently a basic isoelectric point was activated by cysteine and was more active at 65°C than at 35°C. Milk clotting activity increased with pH at 65°C, with the enzyme being almost twice as active at pH 6.4 as at pH 5.4–5.7. Storage at 4°C for 15 days resulted in a 30–50% loss in enzyme activity.

INTRODUCTION

ALTHOUGH THE JUICE from leaves of sodom apple [*Calotropis procera* (Ait.)] has been used over the years in some parts of West Africa for the production of a soft, unripened cheese known as 'Warankasi' in Nigeria or 'Woagachi' in the Republic of Benin, little is known about the milk clotting factor(s) in the leaves. When added to milk in the right proportions, the juice produces a firm curd at high cooking temperatures (Ogundiwin and Oke, 1983; Aworh and Egounlety, 1985). The juice was reported to have the strongest milk clotting activities at temperatures above 70°C at pH 5.6–6.0, with minimal activities at temperatures below 37°C at pH 6.3. Enzyme in the juice demonstrated typical reactions of milk-clotting proteinase of plant origin (Ogundiwin and Oke, 1983).

The purpose of this study was to investigate the extraction conditions and property of a milk clotting enzyme precipitated with ammonium sulfate from sodom apple leaf extract.

MATERIALS & METHODS

Materials

Sodom apple leaves were freeze-dried and milled into a powder. Papain (crude powder, type II) and purified casein were obtained from the Sigma Chemical Co. (St. Louis, MO). Bovine plasma gamma globulin was a product of Bio-Rad Laboratories (Richmond, CA). All other chemicals were reagent grade.

Extraction

Freeze-dried leaves 10–15g were blended with 150–250 mL deionized water in a Waring Blendor for 1 min at 20°C, centrifuged at 13,000 × *g* for 20 min and filtered to obtain the crude extract, pH 5.8–6.0. To investigate the effects of pH on ammonium sulfate precipitation of the enzyme, aliquots (20 mL) of the crude extract were adjusted to various pH values with 1N HCl or 1N NaOH. Solid ammonium sulfate (5–9g) was then added until a noticeable precipitate was formed. After centrifugation at 17,000 × *g* for 10 min, the supernatants were analyzed for residual proteolytic activity and protein.

The extraction method finally adopted was: The crude extract (100 mL) was acidified to pH 3.0 and centrifuged at 13,000 × *g* for 20 min. The resultant precipitate with low enzyme activity was discarded, and the supernatant was brought to pH 9.7 with 1N NaOH, and 40% saturation with solid ammonium sulfate (24g). Following centrifugation, the precipitate was collected and the supernatant brought to 65% saturation with solid ammonium sulfate (19g), and again centrifuged. The resultant precipitate was combined with that from 40% saturation, dissolved in 25 mL 0.02 M phosphate buffer pH 6.5 containing 0.02 M cysteine and dialyzed overnight against the same buffer. All operations were carried out at 4°C.

Protein determination

Protein was determined by the dye-binding method (Bradford, 1976) with bovine plasma gamma globulin as standard.

Enzymatic activities

Proteolytic activity was determined by the casein digestion method (Kunitz, 1947) at 35°C and pH 7.2 using 1% casein in the presence of 5 mM cysteine and 2 mM ethylenediaminetetraacetate (EDTA). A unit of enzyme activity was defined as that which caused an increase of 1 absorbance unit at 280 nm per min.

Milk clotting activity was determined at 35°C by the method of Whitaker (1959). The substrate was a 20% solution of Carnation instant skim milk powder in 0.34M acetate buffer pH 4.6. A unit of enzyme activity clots 1 mL milk in 1 min. Specific activities were expressed as units of activities per mg protein.

All values reported were the averages of at least duplicate tests.

RESULTS & DISCUSSION

THE EFFECT of pH on the precipitation of milk clotting enzyme with ammonium sulfate from sodom apple leaf extract was investigated. Ammonium sulfate precipitation was chosen in this study because of simplicity and economy of the method as discussed by Scopes (1982). Enzyme precipitation was inefficient at low pH and increased with increasing pH. At pH 9.7 the greatest recovery was achieved. Above this pH value, residual proteolytic activity in the supernatant increased. Removal of protein insoluble at pH 3.0 resulted in a twofold increase in the specific activity of the precipitate from 0.013 to 0.028 when precipitated by 65% saturation with ammonium sulfate. Only a slight specific activity of 0.005 (equivalent to the loss of about 3% of the total activity) remained in the precipitate at pH 3.0.

Table 1 shows details of enzyme fractionation from sodom apple leaves. A 1.74-fold increase in specific milk clotting activity was obtained in the final extract, relative to the crude extract. When the crude extract was assayed for proteolytic activity without cysteine (5 mM) and EDTA (2 mM), specific activity was reduced sixfold, from 0.026 to 0.004.

These results suggest that similar to papain (Smith et al., 1954), ficus enzymes (Kramer and Whitaker, 1964) and several other plant proteases, the milk clotting enzyme in sodom apple leaves is a sulfhydryl enzyme, requiring a free sulfhydryl group for its activity, with a basic isoelectric point.

The effect of pH and temperature on the activities of the enzyme recovered from sodom apple leaves by ammonium sulfate precipitation is shown in Fig. 1. The enzyme was more active at 65°C than at 35°C. At 35°C, milk clotting activity decreased with increasing pH from pH 5.0–5.7, followed by a slight increase from pH 5.7–6.4. Proteolytic activity was slightly higher at pH 7.2–8.0 than at 6.3–6.8. The effect of

Author Nakai is with the Dept. of Food Science, Univ. of British Columbia, Vancouver, B.C., Canada V6T 2A2. Author Aworh's present address is Dept. of Food Technology, Univ. of Ibadan, Ibadan, Nigeria.

Table 1—Ammonium sulfate fractionation of milk clotting enzyme from sodom apple

Fraction	Volume (mL)	Total protein (mg)	Specific activities		Total activities	
			Milk clotting	Proteolytic	Milk clotting	Proteolytic
1. Crude extract	100	144.0	0.169	0.026	24.34	3.74
2. Supernatant fluid of acidification to pH 3.0	100	95.0	0.252	0.028	23.94	2.66
3. Precipitate of 0.40–0.65 saturation at pH 9.7	32	37.8	0.294	0.038	11.11	1.44

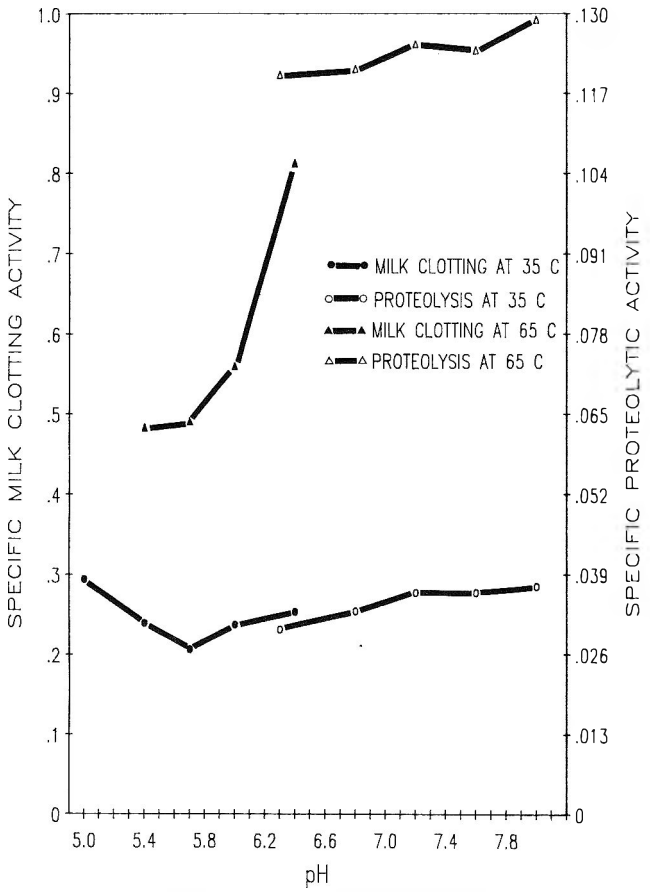

Fig 1—Effect of pH and temperature on milk clotting and proteolytic activities of enzyme from sodom apple. Milk clotting was determined in 20% skim milk—acetate buffer (0.34M) and proteolysis in 1% casein—phosphate buffer (0.2M).

pH on milk clotting activity was more pronounced at 65°C, with the enzyme being almost twice as active at pH 6.4 as at pH 5.4–5.7. Changes in pH had less effect on the proteolytic activity of the enzyme at 65°C.

Our results on the effect of temperature on the activities of the enzyme from sodom apple leaves are consistent with the findings of Ogundiwin and Oke (1983) that the juice from the leaves is more active at higher temperatures. However, contrary to the report (Ogundiwin and Oke, 1983) that the milk clotting activity of the leaf juice decreased with increasing pH, with the optimum at pH 5.6–6.0, we found a greater activity at pH 6.4 than at pH 5.4–6.0 at 65°C (Fig. 1). A similar reaction was observed in the paper of Ogundiwin and Oke (1983) for centrifuged juice from sodom apple leaves. This may explain why, in traditional West African cheesemaking, the juice produces a satisfactory curd without acidification of the milk (Ogundiwin and Oke, 1983; Aworh and Egounlety, 1985).

The effect of storage at 4°C on the proteolytic activity of the enzyme from sodom apple leaves was investigated. Specific proteolytic activity of the crude extract and the ammonium sulfate extract assayed at 35°C decreased from 0.026 and 0.038 to 0.019 and 0.018 after 15 days and 0.015 and 0.016 after 31 days, respectively. The result that 30–50% of enzyme activity was lost after 15 days at 4°C is contrary to the observation of Ogundiwin and Oke (1983) that cold storage at 4°C for 2 weeks did not seem to affect enzyme activity. This is probably due to the difference in method for extraction of enzymes; i.e., they have expressed fresh leaves while freeze-dried leaves have been extracted with water in this study.

With specific milk clotting and proteolytic activities of 7.09 and 1.30, respectively, crude papain was 24 times more active in clotting milk and 34 times more proteolytic than the enzyme from sodom apple leaves at 35°C. It would appear that due to its low proteolytic activity, the enzyme from sodom apple leaves could be a good substitute for rennet for the production of other cheeses, especially those requiring high cooking temperatures.

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A Research Note

Effectiveness of Ethylene Oxide and Gamma Irradiation on the Microbiological Population of Three Types of Paprika

S. LLORENTE FRANCO, J. L. GIMÉNEZ, F. MARTÍNEZ SÁNCHEZ, and F. ROMOJARO

ABSTRACT

The effectiveness of ethylene oxide and the gamma irradiation sterilizing treatments on the microbiological population was studied in three types of Spanish paprika, stored in a cold chamber (4°C) and at room temperature (16–36.8°C) over an experimental period of 285 days. The controlled microorganisms were: mesophilic aerobes, coliforms, sulfite reducing anaerobes, yeasts, molds, and Salmonella. The presence of aflatoxins was also studied. The results showed that both sterilizing treatments reduced the microbiological population to below the permissible levels recommended by the International Commission on Microbiological Specification for Food. Nevertheless, it was interesting that the gamma irradiation treatment was more effective.

INTRODUCTION

SPICES AND HERBS often contain large quantities of microorganisms (Frazier and Werthoff, 1978), which can be contaminating agents in some processed foods. In paprika the microorganisms are mainly mesophilic aerobe bacteria (Julset hand Deibel, 1974) anaerobe sulfite-reducing, coliforms, yeasts, and molds. These microbial populations, as well as the absence of *Escherichia coli* and *Salmonella*, are the main parameters which determine the microbiological quality of paprika, according to the recommendations of the *International Commission on Microbiological Specification for Food* (ICMSF, 1983) for those products containing low moisture levels. Therefore, it is of great interest to investigate the possibility of sterilizing the large quantities of paprika produced annually in Spain using methods based on ethylene oxide and gamma irradiation.

The objective of this study was to investigate the effects of the above mentioned sterilizing treatments on the standard commercial paprikas: "granulated" (water granulated), "added oil" (oil granulated) and "fine grind" (paprika powder), stored in cold chamber (4°C) and at room temperature (16–36.8°C). The influence of the two sterilizing treatments on several parameters determining the paprika quality was also considered.

MATERIAL & METHODS

Plant material

Pepper fruit (*Capsicum annuum* L.) from same crop area, subjected to an industrial drying process, were used for the experiments. After a preliminary treatment to obtain material of the same color range, three commercial samples with those characteristics were used:

granulated type—119.7 ASTA U. color, 11.12% moisture, 13.28% fat extract, and 79.21% fraction retained on a sieve with a 0.150 mm aperture;

added oil type—119.7 ASTA U. color, 6.64% moisture, 20.45% fat extract, and 99.34% fraction retained on a sieve with 0.150 mm aperture;

fine grid type—121.4 ASTA U. color, 7.05% moisture, 15.96% fat extract, and 55.10% fraction retained on a sieve with a 0.150 mm aperture.

From each type, 60 samples of 250g were taken and kept in red thermosealed polyethylene bags for the sterilizing treatments. Control samples (untreated) were also taken. After sterilization some bags were stored in a cold chamber (4°C) and others at room temperature (16–36.8°c) for 285 days. Microbiological and analytical determinations were carried out 15, 35, 55, 75, 105, 135, and 285 days after the sterilization.

Sterilizing treatment

Ethylene oxide (ETO) was used as a mixture of 90% CO_2 and 10% ETO (W/W) in 750 g/m^3 doses over 48 hr at 25–30°C in a sterilizing chamber. Gamma ray irradiation was carried out in the Radioactive Isotopes Dept. of the "Junta de Energia Nuclear" in Madrid (Spain) using 6.5 kGy doses from a ^{60}Co source.

Storage

A cold chamber (4°C temperature and 60% relative humidity) was the most suitable for maintaining the paprikas sensory characteristics (Giménez et al., 1984). Room temperature ranged between 16°C and 36.8°C with relative humidity varying from 41.2 to 49%.

Microbiological procedures

The procedures proposed by American Spices Trade Association in *Official Microbiological Methods* (ASTA, 1976) were followed: mesophilic bacteria count, M.2.0; coliforms bacteria, M.3.1; *E. coli* identification, M.3.4; Salmonella, M.8.0; yeasts and molds, M.4.0; sulfite-reducing count according to Pascual Anderson (1982).

Analytical methods

Analyses were made according to ASTA (1985) methods: moisture, M.2.0; color, extraction of capsicum and oleoresins, M.20.1; fat, methylene chloride extraction, M.11.0; sieve analysis, M.10.0, and aflatoxins according to a modification of the method of AOAC (1980), as proposed by Romojaro et al. (1982).

RESULTS & DISCUSSION

THE EVOLUTION of the microbiological populations in three paprika types for the two sterilization treatments and control, stored at room temperature (16–36.8°C) and in the cold chamber (4°C), are shown in Tables 1 and 2. These tables show that Salmonella and *E. coli* were absent in all analyzed samples.

The mesophilic aerobes population of the untreated samples, that was mainly composed of aerobic microorganisms, increased in both storage systems. The control population whose initial count ranged between 4.9×10^6 and 7.0×10^6 per gram increased over the storage period. This effect was greater for the samples stored at 16–36.8°C.

The ethylene oxide doses and the exposure time employed in the sterilizing treatment can be considered as suitable since eradication reached 97% after 55 days at 16–36.8°C and 4°C. The resulting counts were within the permissible range proposed by ICMSF (1985). When the samples were stored as 4°C, the efficiency of this sterilizing treatment was greater than when the samples were kept at 16–36.8°C. Only the granulated paprika type reached counts higher than the permissible levels

—Text continued on page 1574

The authors are affiliated with Centro de Edafología y Biología Aplicada del Segura. C.S.I.C. Murcia. España.

Table 1—Effects of ethylene oxide and gamma irradiation on the microbiological content of paprika (count/g) [Cold chamber (4°C)]

Microorganisms	Treatment	Days from treatments						
		15	35	55	75	105	135	285
		Type: GRANULATED						
Mesophilic aerobes	Control	4.9×10^6	10.5×10^6	11.2×10^6	12.5×10^6	20.8×10^6	19.6×10^6	20.0×10^6
	ETO	1.4×10^5	2.9×10^5	3.0×10^5	3.1×10^5	9.0×10^5	1.5×10^6	1.3×10^6
	Gamma	10000	10000	10000	15000	20000	20000	40000
Coliforms	Control	4300	6000	7500	11000	11000	9300	4600
	ETO	—	—	—	—	—	—	—
	Gamma	—	—	—	—	—	—	—
Sulfite reducing	Control	5200	18000	20000	25000	7000	6000	500
	ETO	—	—	—	—	—	—	—
	Gamma	—	—	—	—	—	—	—
Yeasts and molds	Control	9000	16000	18000	21000	17000	14000	8000
	ETO	<100	<100	100	<100	100	200	100
	Gamma	100	100	<100	300	<100	400	400
		Type: ADDED OIL						
Mesophilic aerobes	Control	5.7×10^6	9.6×10^6	9.0×10^6	16.5×10^6	17.7×10^6	19.5×10^6	18.5×10^6
	ETO	3.0×10^5	3.5×10^5	3.9×10^5	4.2×10^5	4.5×10^5	4.3×10^5	4.4×10^5
	Gamma	40000	40000	40000	60000	60000	50000	50000
Coliforms	Control	2400	5300	7200	9500	19000	2900	2400
	ETO	—	—	—	—	—	—	—
	Gamma	—	—	—	—	—	—	—
Sulfite reducing	Control	5600	10000	11000	13000	10000	5000	1800
	ETO	—	—	—	—	—	—	—
	Gamma	—	—	—	—	—	—	—
Yeasts and molds	Control	6200	9000	12000	18000	12000	10000	6400
	ETO	900	900	700	700	700	1000	1000
	Gamma	100	100	500	500	600	600	600
		Type: FINE GRIND						
Mesophilic aerobes	Control	7.0×10^6	14.2×10^6	17.7×10^6	20.5×10^6	20.8×10^6	20.4×10^6	23.0×10^5
	ETO	1.0×10^5	1.1×10^5	1.2×10^5	2.0×10^5	2.2×10^5	3.5×10^5	3.7×10^5
	Gamma	10000	13000	15000	20000	28000	28000	
Coliforms	Control	2300	2900	4600	4300	11000	11000	1600
	ETO	—	—	—	—	—	—	—
	Gamma	—	—	—	—	—	—	—
Sulfite reducing	Control	7200	9600	15000	20000	6000	5000	1000
	ETO	—	—	—	—	—	—	—
	Gamma	—	—	—	—	—	—	—
Yeasts and molds	Control	8500	32000	40000	36000	27000	19000	16000
	ETO	1600	1500	1200	1500	3200	2500	2100
	Gamma	100	100	300	400	500	500	500

Table 2—Effects of ethylene oxide and gamma irradiation on the microbiological content of paprika. (count/g). [Room temperature (16–36.8°C)]

Microorganisms	Treatment	Days from treatments				
		55	75	105	135	285
		Type: GRANULATED				
Mesophilic aerobes	Control	18.4×10^6	24.0×10^6	22.8×10^6	24.8×10^6	22.0×10^6
	ETO	5.0×10^5	6.6×10^5	9.5×10^5	1.8×10^6	1.7×10^6
	Gamma	30000	32000	38000	40000	50000
Coliforms	Control	9200	12000	15000	15000	16000
	ETO	—	—	—	—	—
	Gamma	—	—	—	—	—
Sulfite reducing	Control	8000	2000	1000	500	100
	ETO	—	—	—	—	—
	Gamma	—	—	—	—	—
Yeasts and molds	Control	9000	12000	11000	11000	7000
	ETO	<100	<100	<100	<100	<100
	Gamma	<100	100	<100	<100	300
		Type: ADDED OIL				
Mesophilic aerobes	Control	11.9×10^6	21.9×10^6	22.8×10^6	23.6×10^6	24.0×10^6
	ETO	5.0×10^5	6.7×10^5	6.5×10^5	6.0×10^5	6.4×10^5
	Gamma	70000	100000	100000	100000	100000
Coliforms	Control	7500	11000	21000	24000	24000
	ETO	—	—	—	—	—
	Gamma	—	—	—	—	
Sulfite reducing	Control	9000	2000	1000	1000	100
	ETO	—	—	—	—	—
	Gamma	—	—	—	—	—
Yeasts and molds	Control	5000	15000	10000	8000	6000
	ETO	200	200	200	200	300
	Gamma	200	100	300	200	300
		Type: FINE GRIND				
Mesophilic aerobes	Control	20.0×10^6	21.4×10^6	23.5×10^6	25.2×10^6	29.0×10^6
	ETO	1.6×10^5	2.7×10^5	3.8×10^5	4.9×10^5	5.1×10^5
	Gamma	22000	25000	25000	32000	33000
Coliforms	Control	9300	15000	24000	24000	23000
	ETO	—	—	—	—	—
	Gamma	—	—	—	—	—
Sulfite reducing	Control	5000	3000	3000	2000	100
	ETO	—	—	—	—	—
	Gamma	—	—	—	—	—
Yeasts and molds	Control	20000	28000	25000	17500	15500
	ETO	600	200	200	600	500
	Gamma	300	100	100	400	400

A Research Note

Stability Evaluation of Oil-in-Water Emulsions by Gold Sol

APURBA K. RAY and JOHN K. JOHNSON

ABSTRACT

A simple, rapid, and inexpensive titration method with gold sol was developed which measured the fraction of hydrophilic gum arabic that was free in the aqueous (continuous) phase and thus indirectly, the fraction of the gum which coated the oil droplets, thereby fulfilling its intended stabilizing function. For a series of gum arabic-stabilized emulsions of varying particle sizes, the gold number measured for fresh emulsions related directly to the long term storage stability of these emulsions at high dilution.

INTRODUCTION

AN EMULSION is inherently unstable. No single criterion of emulsion instability is sufficient. Among the more commonly used are the decrease in number of droplets with time and visible separation (Vold et al., 1978). To minimize the time required to evaluate emulsion products, many techniques have been tried, with varying degrees of success, to follow the instability under the influence of accelerated aging (Sherman, 1971; Roehl, 1972).

The object of this study was to develop a simple, rapid, and inexpensive method for predicting the relative stability of oil-in-water emulsions. Results obtained with this titration method using gold sol compared well with other stability tests.

MATERIALS & METHODS

Materials

Materials and their sources for emulsions have been given elsewhere (Ray et al., 1983). Sodium chloride (A.C.S. certified) and colloidal gold sol were obtained from Fisher Scientific Co. (Fairlawn, NJ).

Emulsions

Emulsions of varying degrees of dispersion, hereinafter referred to as "original" emulsions, were prepared: Oil part, 240g orange flavor (>99.5% w/w orange oil), 30g brominated vegetable oil, and 100g ester gum; water solution, 912g gum arabic and 2718g water.

The blended oil part was added to the water solution with continuous stirring, and the mixture was homogenized in a Gaulin M3 homogenizer (Gaulin Corp., Everett, MA). The first and second stages of homogenization conditions used for preparing the emulsions were: emulsion #1—3600 psi/400 psi, 1 pass; #2—4000/500, 1 pass; #3—3800/700, 1 pass; #4—3600/400, 2 passes; #5—4000/500, 2 passes. No change was observed in any of these fresh original emulsions upon centrifugation at 700 × *g* for 30 min.

Measurements

Gold number determination. Gold number is defined as the amount (in mg) of the protective hydrocolloid that is just insufficient to prevent a color change of 10 mL of a red gold sol to violet when 1 mL of 10% w/v NaCl is added (Zsigmondy, 1917). The dilute emulsions and gum arabic solution used for titration with gold sol always contained 10 mg gum/mL (Crockford et al., 1975).

Stability index determination. The original emulsions were diluted 1500-fold with water and absorbances were determined at 700 and 400 nm against water (Ray et al., 1983). The ratio of absorbances at 700 and 400 nm will be called stability index (Horie et al., 1978; Kaufman and Garti, 1981).

Interfacial area and droplet size determination. The method has been given elsewhere (Ray et al., 1983).

Storage study. Original emulsions #1—5 were diluted (diluent: 12.4% sucrose, 0.176% citric acid and 0.021% sodium benzoate, all in w/w) and stored (at 21–22°C) upright and undisturbed in Babcock bottles (50-fold dilute; 50 mL) for up to 37 days and beverage bottles (500-fold dilute; 300 and 950 mL; flint glass) up to 140 days, respectively.

RESULTS & DISCUSSION

GUM ARABIC, an anionic macromolecular polysaccharide, is a well known emulsifier (Glicksman, 1983). Gold sol, being (negatively) charged, is susceptible to coagulation and thus changes color upon addition of electrolytes (here, NaCl). This susceptibility to coagulation (or aggregation) is reduced due to electrical and steric stabilization when the anionic gum is adsorbed upon addition to gold sol (Pugh and Heller, 1960; Tadros, 1982).

In an emulsion, some fraction of the total (emulsifying) gum is adsorbed at the surface of the oil droplets and the rest stays as free gum molecules. Considering (a) that the gold sol particles are much smaller than emulsion droplets, (b) the steric restriction imposed upon the gum molecules adsorbed onto the oil droplets, and (c) the likelihood that the part of the gum molecule that can adsorb at the oil droplet surface is the same part that would adsorb at the surface of gold sol particles (both surfaces being hydrophobic), it seems quite unlikely that the oil-surface adsorbed gum molecules can provide protection to the gold sol to any significant extent.

Thus, if the free hydrocolloid plays the only important role in stabilizing gold sol, these titration results can be considered a measure of the fraction (or amount) of the total gum that is free, the rest being adsorbed onto the oil droplets. As an example, gold numbers for the gum arabic standard solution and emulsion #5 were 0.2 and 0.7, respectively (see Table 1). Since both the solutions were equal in total gum concentration (i.e., 10 mg/mL), we can conclude that 70 μL of diluted emulsion #5 contained the same amount of free gum as in 20 μL gum solution. It follows that about 29% of the total gum was free in the "original" emulsion #5 and the rest adsorbed onto the oil droplets. Implicit in this calculation was the assumption that within the short time for emulsion dilution and titration, no significant amount of gum had desorbed from the oil droplets (Koral et al., 1958; Ray et al., 1983).

The results show (Table 1) that the gold number correlated fairly well with the long term stability of dilute emulsions and the stability index. A direct relationship between the specific interfacial area of emulsion droplets and gold number (Table 1) was found. As the interfacial area increases, more gum is expected to be adsorbed (Ray et al., 1983). Consequently, less

Authors Ray and Johnson are with Corporate Research and Development Dept., The Coca-Cola Company, Atlanta, GA 30301.

Table 1—Stability index and gold number of emulsions of a given composition

Emulsion	Specific interfacial area (m^2/cm^3)	Geometric mean diameter (μm)	Stability index (A_{700nm}/A_{400nm})	Gold no.
# 1	4.83	1.30	0.453	0.4
# 2	7.27	0.87	0.360	0.5
# 3	10.11	0.62	0.320	0.5
# 4	11.26	0.56	0.233	0.6
# 5	11.99	0.52	0.250	0.7
Stability of emulsions on the basis of:				
Gold number:				#5>4>3≈2>1
Stability index:				#4>5>3>2>1
Beverage bottle storage:				#5≈4>3>2>1
Babcock bottle storage:				#4>5>3>2>1

free gum would be available to protect the gold sol and thus, the increase in gold number due to additional gum that must be added to stabilize the gold sol.

Calibration of gold sol with a standard gum solution would make it possible to compare gold numbers of emulsions measured at different times with different batches of gold sol. This method can be used for comparing the stabilities of a series of oil-in-water emulsions prepared with any particular emulsifying hydrocolloid (anionic or nonionic). The titration method was also found to be applicable with colored emulsions. Depending upon the nature of the emulsion and end-use, other suitable and relevant accelerated test(s) should be performed in conjunction with gold sol titration to arrive at a high level of confidence in predicting emulsion stability.

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105 days after the treatment, possibly because of its higher moisture and particle aggregation. A higher dose of ethylene oxide or a greater exposure period might be necessary. The most efficient degree of eradication for this aerobic flora was achieved by the gamma irradiation treatment, which produced a 99.8% decrease relative to the initial population 285 days after the treatment.

The initial count of coliform (MPN) in the control samples ranged between 2300 and 4300 per gram of paprika. These figures were higher than the maximum suggested by ICSMF (1985). These populations increased and reached values of 11000 for granulated and fine grind, and 19000 for added oil, under cold chamber storage conditions at 105 days. After this the coliform MPN counts showed a decreasing trend, and at the end of experimental period (285 days after treatments), reached a count of 4600 for the most contaminated sample. Nevertheless, the counts for the samples stored at 16–36.8°C increased continuously. The highest MPN count corresponded to the added oil type.

Similar trends were shown by sulfite-reducing anaerobe populations, whose control sample values were initially higher than permissible levels. These decreased with time and were greatest in the samples stored at room temperature. These microorganisms were fully eradicated by both sterilizing treatments. Therefore, the conditions used may be considered optimal.

The yeast and mold contents are of great interest because some species, under suitable environmental conditions, may give rise to certain mycotoxins which present danger for human health. Analysis of the control samples of the paprika types gave microbial counts higher than the permissible maximum range, though aflatoxins were not detected in these samples. The evolution of trend was similar to the above cases, with a maximum at 75 days after treatments. There were no significant differences between the two storage conditions. As before, the sterilizing treatments were effective for this microbiological fraction, with suitable results for both procedures. Ethylene oxide was most efficient when used with the fine grind type for which decreases of 99.1% were obtained. These results are in agreement with those obtained by Mayr and Suhr (1973), who concluded that a certain moisture level is needed to achieve the best result with this treatment which was also most effective for the three types of paprika as the resulting counts were always lower than the permissible levels proposed by the International Commission on Microbiological Specification for Food. (ICMSF, 1985).

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A Research Note

Moisture Sorption Studies on Garlic Powder

NAZAR MOHAMMAD, M. ANWAR, A.F.M. EHTESHAMUDDIN, and MOHAMMAD JAMIL

ABSTRACT

The moisture sorption isotherms of two samples of garlic powder, one containing corn starch (CS) and the other without starch, were determined using saturated salt solutions. Monolayer values were calculated from the isotherms using the Brunauer, Emmett and Teller (BET) equation. The addition of CS at 5% slightly increased the monolayer value of the garlic powder, but it had little effect on the caking behavior of the powder. Equilibrium relative humidity (ERH) of garlic powder corresponding to the monolayer value was found to be 14%.

INTRODUCTION

FOR A SYSTEMATIC STUDY on the packaging and storage behavior of dehydrated foods, the measurement of equilibrium relative humidity (ERH) is of considerable importance. The ERH may be defined as the percent ratio of vapor pressure of a dehydrated food to the vapor pressure of pure water at the same temperature as the food. It is numerically equal to the water activity of the food expressed as a percentage. The ERH of a food material determines whether it will gain or lose moisture in a particular environment and so this property is more relevant to the storage behavior than is the moisture content (Taylor, 1961).

A knowledge of the sorption isotherms or ERH curves of dehydrated food products is important for predicting the quality stability during storage (Labuza, 1968). The moisture at which certain dehydrated foods have good storage stability has been found to agree closely with the moisture representing a calculated monolayer of adsorbed water (Salwin, 1959). This monolayer can be calculated from moisture sorption data by means of the Brunauer, Emmett and Teller (BET) theory (Brunauer et al., 1938). Moisture sorption isotherms of various food products have, therefore, been investigated by many researchers (Agrawal and Clary, 1971; Berlin et al., 1968; Berry and Dickerson, 1973; Gane, 1950; Heldman, et al., 1965; Hayakawa et al., 1978; Kilara and Humbert, 1972; Quast and Teixeira Neto, 1976; Rasekh et al., 1971).

Dehydrated garlic has great commercial value on account of its culinary properties, but moisture sorption data on garlic powder are very rare in the published literature. This paper reports on the study of sorption characteristics of garlic powder produced on a commercial scale.

MATERIALS & METHODS

GARLIC POWDER used in our experiments was prepared by drying fresh garlic in a tunnel dehydrator, followed by dehusking and milling to yield a 30 mesh product (Jamil and Ehteshamuddin, 1980). The sample was divided into two portions. Corn starch (CS), 5% (w/w), was mixed thoroughly with one portion of the garlic powder to see if it had an anti-caking effect while the other portion used was without starch. The mositure of both samples was determined by the vacuum oven method (AOAC 1970).

Moisture sorption isotherms were determined by the method described by Hayakawa et al. (1978) for coffee products with a slight modification. Duplicate samples, each of about 2g, were placed at 30 ± 1°C over saturated salt solutions in separate desiccators. The salts used were lithium chloride, potassium acetate, chromium trioxide, magnesium nitrate, sodium acetate, ammonium sulfate and ammonium dihydrogen phosphate. The relative humidity (RH) data of these salts are given by Wink (1946). The samples were weighed on alternate days and when the two consecutive weighings were equal, it was assumed that equilibrium condition was reached. Equilibrium moisture of each sample was determined by the vacuum oven method (AOAC, 1970). Physical appearance of the samples with respect to color, flowability was also observed.

Monolayer values for moisture were calculated as described by Salwin (1963). The isotherm in the RH range 5–35% was transformed to give a straight line BET plot. From the BET plot, intercept 'I' and slope 'S' were found and monolayer value 'a_1' (g H_2O/100 g dry material) was calculated from the following equation:

$$a_1 = 1/(I \times 100S)$$

The tabulated values were analyzed through the application of analysis of variance technique (Snedecor and Cochran, 1967) to examine the influence of CS, RH and the interaction of these factors (CS × RH).

All authors are with the Food Technology & Fermentation Division, PCSIR Laboratories, Lahore-16, Pakistan.

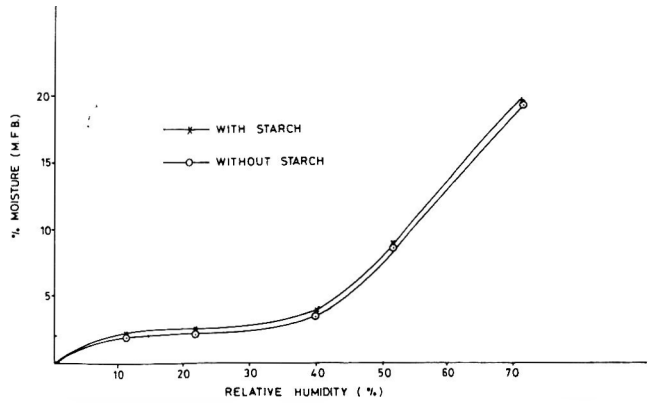

Fig. 1—Moisture sorption isotherms for garlic powder at 30°C.

RESULTS & DISCUSSION

THE RELATIONSHIP between equilibrium moisture content, days required to reach equilibrium at various relative humidities, and physical appearance of garlic powder with and without starch is presented in Table 1. Sorption isotherm curves of the two samples are shown in Fig. 1.

The results in Table 1 show that both samples required the same time for equilibration at any RH. Moisture at equilibrium was slightly more in the sample containing starch but there was no marked difference in the appearance of both the samples. Considering the free flowing property and appearance of the garlic powder, the appropriate RH for its storage appeared to be below 40%. The time required to reach equilibrium was found to decrease with a decrease in RH and then increase at very low values of RH. This is in accordance with the results recorded by Bhatia and Amin (1962) on dehydrated tropical fruits. The difference, however, lies in the fact that whereas the fruits required more time to reach equilibrium in lower ranges of RH, garlic powder did so in the higher ranges of RH. This difference in moisture sorption pattern may be attributed to the difference in the chemical composition and the

Table 1—Relation of equilibrium moisture, time required to reach equilibrium, and appearance of garlic powder exposed to various relative humidities at 30°C

Salt used	Relative humidity (%)	Sample	Days required for equilibration	Equilibrium moisture (%) MFB[a]	Remarks
Ammonium dihydrogen phosphate	92.0	Without starch[b]	–	–	Moldy after 22 days; flowability reduced after 3rd day.
		With starch	–	–	
Ammonium sulfate	79.6	Without starch	37	20.13	Flowability reduced after 4th day; caking was in an advanced stage after 30 days and color slightly brownish and dull.
		With starch	37	21.99	
Sodium acetate	71.4	Without starch	33	19.16	Flowability affected after 4th day. Caking in advanced stage; color brownish and dull.
		With starch	33	19.50	
Magnesium nitrate	51.4	Without starch	24	8.57	Free flowing property markedly reduced; appearance good.
		With starch	24	8.81	
Chromium trioxide	40.0	Without starch	13	3.44	Free flowing; appearance good.
		With starch	13	3.73	
Potassium acetate	22.0	Without starch	20	2.20	Free flowing; appearance excellent.
		With starch	20	2.45	
Lithium chloride	11.2	Without starch	30	1.80	Free flowing, appearance excellent.
		With starch	30	2.15	

[a] MFB = moisture free basis.
[b] Initial moisture content (%, MFB): without starch, 2.67; with starch, 2.75.

particle or piece size of the two products. Caking started in both samples at about 9% moisture (51.4% RH) which is close to that reported by Pruthi et al. (1959) who found that 10.6% moisture is the critical point for caking of garlic powder.

The isotherm curves (Fig. 1) are typical S-shaped and indicate that the equilibrium moisture increases very slowly with an increase in environmental RH up to 40% RH, beyond which there is a steep rise in moisture in both samples. Addition of CS to garlic powder increased its equilibrium moisture at all levels of RH studied. According to the statistical analysis, CS and RH factors were found to affect equilibrium moisture significantly ($P < 0.1$). The effect of CS × RH interaction, however, was not significant.

The monolayer value (a_1), i.e., the optimum moisture calculated from the sorption isotherms was 2.0% for garlic powder and 2.25% for the garlic powder containing CS. The increase in the monolayer value caused by the addition of 5% CS, though statistically significant, had no considerable effect on the flowability and caking behavior of the garlic powder as evidenced in Table 1.

ERH of garlic powder corresponding to its monolayer value of 2% may be obtained from Fig. 1 and is about 14%. This is, in fact, the level at which garlic powder keeps very well on storage for a longer period of time. Our results in this respect agree with those reported by Pruthi et al. (1959).

It may be concluded that garlic powder belongs to a class of food powders that are highly hygroscopic by nature and that addition of 5% CS has little effect on the caking properties.

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A Research Note

Factors Governing the Greening of Garlic Puree

T. M. LUKES

ABSTRACT

Storing garlic bulbs for a month at or above 23°C, prior to processing, prevented the production of a green pigment in the garlic puree. The amino acid S-(1-propenyl) cysteine sulfoxide was necessary for the development of the green color.

INTRODUCTION

GARLIC PUREE has been a commercial food product for over forty years, produced by breaking garlic bulbs into cloves, cleaning and then grinding them. The grinding is done in a manner that results in a skin-free garlic puree. Sodium chloride is usually added and citric acid is always added. The final pH value is near 4. The temperature is then raised in a heat exchanger to inactivate the enzymes and reduce the number of microorganisms. Ideally, the final product should have a light tan to cream color. Some lots of garlic bulbs produce purees which are dark green to blue-green instead of cream (Sano, 1950; Shannon, 1961). This product is unsaleable because of the green color, the cause of which is unknown. The object of this study was to find a method of preventing the development of the green pigment.

MATERIALS & METHODS

Testing for puree color

Garlic cloves were hand peeled and pureed in a food blender with twice their weight of water and enough citric acid to reduce the pH value to between 4.0–4.3. This puree was held over night at 45°C, filtered and the amount of green color was determined at 590 nm.

Amino acid analysis

The garlic was prepared for analysis by peeling the cloves and then slicing them in half lengthwise. Twenty grams of the halves were ground in a kitchen blender for 2 min with 60g of water containing 1.6 mL of 5N HCl and 40 mg of cysteic acid, as an internal standard. The HCl was added to reduce the pH value to 1.8 to 2.0. At this value the allinase was not able to react with the sulfoxides. It was assumed that unheated solutions at pH 2 would not cause these amino acids to hydrolyze to any great extent. The puree was filtered, and an aliquot was placed directly on a 0.9 by 40 cm column packed with Dowex 50-X8. The solvent was 0.2M sodium citrate buffer (pH 2.9) with 10% methanol added. After the first 40 mL of buffer was run through the column, the pH was slowly raised by adding increasing amounts of 0.2M, pH 8.2 sodium citrate buffer to the reservoir. The amino acids were separated by the above ion exchange chromatography with post column analysis with ninhydrin (Moore et al., 1958).

Storage tests

Commercially grown California Late and California Early cultivars, purchased in a local market, were stored at four different temperatures: 3°C, 12°C, 23°C, and 28°C. Periodically, samples were withdrawn and tested for the amount of green pigment that would develop when pureed.

Author Lukes is with the Food Science and Human Nutrition Dept., California Polytechnic State Univ., San Luis Obispo, CA 93407.

Effect of S-(1-propenyl) cysteine sulfoxide (PECSO) on color

Ten mL of puree were mixed with various amounts of a solution of PECSO in pH 4.0, 0.2M citric acid buffer. More buffer was added to make a final volume of 15 mL. The mixture was held overnight at 45°C, filtered and the green pigment measured at 590 nm in 1 cm cells with a Bausch and Lomb Spectronic 21 spectrophotometer. PECSO was prepared from onion by the method of Carson et al. (1966).

RESULTS & DISCUSSION

THE COLOR DEVELOPED with the garlic puree stored at various temperatures is shown in Table 1. The California Early cultivar did not develop green color initially but did so to an increasing degree when stored at or below 12°C. Above 23°C the color was still satisfactory after 32 days storage. The California Late cultivar had an initial light green color which in-

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Table 1—Color of the garlic puree after storage of the bulbs at the temperatures indicated

Cultivar	Temp (°C)	Days stored 0	18	32
California Early	3	cream	lt green	dark blue-green
Early	12	cream	lt green	blue-green
Early	23	cream	cream	cream
Early	28	cream	cream	cream
California Late	3	lt green	lt green	dark blue-green
Late	12	lt green	lt green	blue-green
Late	23	lt green	cream	cream
Late	28	lt green	cream	cream

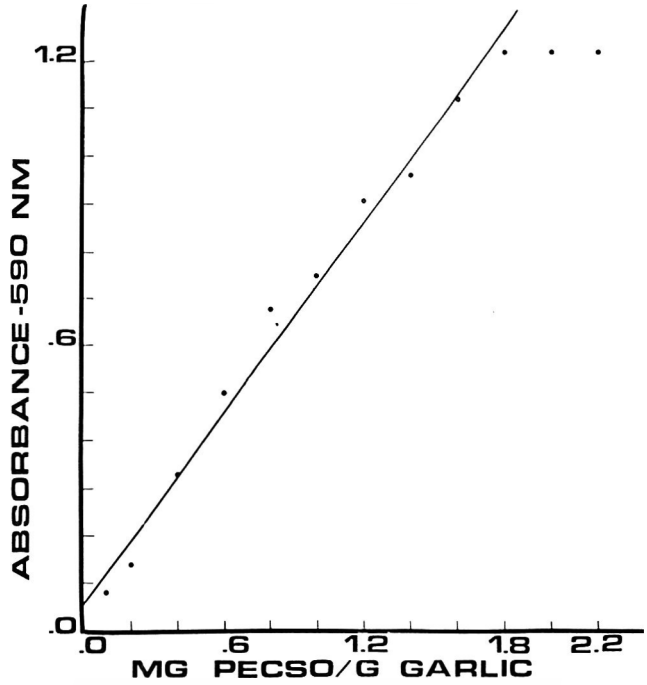

Fig. 1—Relationship between absorbance at 590 nm and mg of S-(1-propenyl) cysteine sulfoxide (PECSO)/g of garlic.

A Research Note

A Quality Comparison of Devil's Food and Yellow Cakes Baked in a Microwave/Convection Versus a Conventional Oven

C. T. STINSON

ABSTRACT

A quality comparison was made of devil's food and yellow cakes baked in microwave/convection (MW/C) and conventional (CON) ovens as either single or double layers. Single MW/C layers were rated slightly lower than CON layers for crust color, moistness and total sensory score. However, double layers were rated equal or near-equal to CON layers. Although slight differences were found, all MW/C cakes were acceptable. These results indicated that high quality cakes can be baked 15–25% faster in a MW/C than in a CON oven.

INTRODUCTION

BAKING with microwave energy is faster than conventional means, but the resultant cakes have less acceptable crust color, moistness, and shape (Hill and Reagan, 1982; Lorenz et al., 1973; Martin and Tsen, 1981; Neuzil and Baldwin, 1962; Proctor and Goldblith, 1948; Street and Surratt, 1961). However, combination microwave/convection (MW/C) ovens are rapid and can brown bakery products, but data on their quality are limited to our assessment of MW/C cakes using different baking conditions (Stinson, 1986). The objective of this research was to extend this study by comparing the quality of devil's food and yellow cakes baked in a MW/C versus a conventional (CON) oven.

MATERIALS & METHODS

DEVIL'S FOOD and yellow cakes were prepared as described previously (Stinson, 1986). In each experiment, two single and two double layers were baked in a MW/C oven (Sharp Model R8320, Sharp Electronics Corporation, Paramus, NJ) and two double layers in a CON oven (General Electric Model J794, General Electric Company, Louisville, KY). The single layers were placed on the bottom shelf of the MW/C oven, whereas the double layers were positioned on the top and bottom shelves in each oven.

The cakes were baked in 8 in (20.3 cm) shiny aluminum pans in preheated ovens. The baking temperatures were 177°C for the CON oven, and 163°C with 10% microwave power for the MW/C oven. The baking times in the CON oven were 30 and 25 min, whereas the times in the MW/C oven were 13 and 14 min for single layers, and 20–22 and 19–21 min for double layers, for devil's food and yellow cakes, respectively.

The sensory and objective evaluation methods were described previously (Stinson, 1986). The data for devil's food and yellow cakes were analyzed separately using analysis of variance, followed by the "Q" test (Snedecor and Cochran, 1980).

RESULTS & DISCUSSION

Moisture

The MW/C cakes had stickier crusts and drier crumb than the CON cakes (Table 1). Moisture of MW/C crusts was 2.2–3 percentage points higher than CON crusts, except for double layer MW/C devil's food crusts, which were not significantly different. On the other hand, moisture of MW/C crumb was 1.7–2.7 percentage points lower than CON crumb.

Previous studies reported that moisture of cakes baked by microwaves only also was less than that of conventionally baked cakes when measured by sensory evaluations or moisture weight loss (Neuzil and Baldwin, 1962; Street and Surratt, 1961).

Symmetry, volume, uniformity and shrinkage indices

Double MW/C devil's food layers had a higher symmetry index than single MW/C or CON layers (Table 1). They had rounded tops whereas the other layers were almost flat. In contrast, the symmetry index of all MW/C yellow layers was lower than that of CON layers. Thus, MW/C yellow layers were flatter than CON layers.

The volume, uniformity and shrinkage indices were not affected by the type of oven, except for single MW/C yellow layers. The volume index was lower and the uniformity index was higher for these layers, resulting in a less desirable appearance. In contrast, several researchers found that white or yellow cakes baked by microwaves only had larger volumes and irregular surfaces compared to conventionally baked cakes (Neuzil and Baldwin, 1962; Street and Surratt, 1961). Although Hill and Reagan (1982) found no differences in the volume of yellow cakes baked in the two ovens, the microwave cakes received lower appearance scores due to tunnels and uneven surfaces.

Penetrometer values

The penetrometer values of the cakes baked in the two ovens were not significantly different (Table 1). These results agreed with the sensory scores for tenderness. However, cakes baked by microwaves only have been reported to be less tender than conventionally baked cakes when measured objectively by shear values, compressibility or breaking strength (Neuzil and Baldwin, 1962; Hill and Reagan, 1982).

Color

The MW/C devil's food cakes had lower "a_L" (redness) and "b_L" (yellowness) scores for crust color than the CON cakes, but no differences were found in "L" (lightness) scores (Table 2). Similar differences in the redness of devil's food cakes baked in a microwave versus a CON oven have been reported (Neuzil and Baldwin, 1962).

Both single and double MW/C yellow layers had higher "L" and "b_L" scores for crust color than the CON layers. In addition, the single MW/C yellow layers had lower "a_L" scores. Also, less browning occurred in single layer crusts due to shorter cooking times and less exposure to dry convective heat. Differences in crust color were larger for single than double devil's food and yellow layers, and resulted in lower sensory scores. No differences were found in crumb color due to oven type.

Author Stinson is affiliated with the Foods & Nutrition Program, Dept. of Health, Physical Education, & Recreation, Univ. of Louisville, Louisville, KY 40292.

Table 1—Moisture content, symmetry, volume, uniformity and shrinkage indices, and penetrometer values of cakes baked in a conventional or a microwave/convection oven[z]

Type of cake and oven	Moisture content (%)		Symmetry index (cm)	Volume index (cm)	Uniformity index (cm)	Shrinkage index (cm)	Penetrometer values (mm)
	Crust	Crumb					
Devil's Food Cake							
Conventional							
Double Layers	26.9 ± 0.7a	40.7 ± 0.5a	0.37 ± 0.13a	13.70 ± 0.19a	0.14 ± 0.04a	1.25 ± 0.06a	19.5 ± 0.2a
Microwave/Convection							
Single Layers	29.9 ± 0.6b	38.0 ± 0.5b	0.35 ± 0.10a	13.23 ± 0.11a	0.23 ± 0.05a	1.43 ± 0.04a	19.4 ± 0.6a
Double Layers	27.7 ± 0.3a	38.8 ± 0.4b	1.00 ± 0.16b	13.55 ± 0.03a	0.10 ± 0.01a	1.31 ± 0.03a	19.6 ± 0.2a
Yellow Cake							
Conventional							
Double Layers	26.2 ± 0.4c	36.1 ± 0.2c	0.76 ± 0.09c	12.81 ± 0.27b	0.18 ± 0.05b	1.49 ± 0.04b	19.8 ± 0.6b
Microwave/Convection							
Single Layers	28.4 ± 0.3d	33.8 ± 0.1d	0.37 ± 0.09d	11.93 ± 0.15c	0.42 ± 0.09c	1.65 ± 0.05b	18.8 ± 0.6b
Double Layers	28.5 ± 0.2d	34.4 ± 0.3e	0.54 ± 0.05e	12.33 ± 0.07b	0.14 ± 0.04b	1.53 ± 0.03b	19.4 ± 0.4b

[z] The values are expressed as mean ± standard error of the mean, n = 4. Means within columns followed by the same letter are not significantly different at the 5% level.

Table 2—Hunter color values and sensory scores of cakes baked in a conventional or a microwave/convection oven[x]

Type of cake and oven	Crust color[y]			Sensory scores[z]		
	L	a_L	b_L	Moistness	Crust color	Total
Devil's Food Cake						
Conventional						
Double Layers	21.17 ± 0.61a	10.14 ± 0.18a	6.19 ± 0.27a	8.2 ± 0.1a	9.7 ± 0.2a	92.9 ± 0.6a
Microwave/Convection						
Single Layers	19.46 ± 0.24a	7.38 ± 0.19b	4.80 ± 0.09b	7.0 ± 0.3b	5.4 ± 0.2b	88.9 ± 0.5b
Double Layers	20.38 ± 0.68a	8.53 ± 0.26c	5.30 ± 0.16b	8.9 ± 0.3a	9.2 ± 0.2a	91.7 ± 0.5a
Yellow Cake						
Conventional						
Double Layers	44.34 ± 0.59b	14.44 ± 0.27d	20.06 ± 0.19c	9.5 ± 0.2c	8.8 ± 0.3c	97.5 ± 0.8c
Microwave/Convection						
Single Layers	58.05 ± 0.60c	11.69 ± 0.18e	30.85 ± 0.54d	8.4 ± 0.1d	7.5 ± 0.3d	92.0 ± 0.4d
Double Layers	50.60 ± 0.96d	14.33 ± 0.13d	25.21 ± 0.22e	9.1 ± 0.2c	8.5 ± 0.2c	94.1 ± 0.6e

[x] The values are expressed as mean ± standard error of the mean, n = 4. Means within columns followed by the same letter are not significantly different at the 5% level.

[y] Color values: L = lightness, 100 = white, 0 = black; a_L = redness; b_L = yellowness.

[z] Total score is 110, based on cell uniformity (10 pts.), cell size (10 pt), cell wall thickness (10 pt), grain (16 pt), moistness (10 pt), tenderness (14 pt), softness (10 pt), crumb color (10 pt), crust color (10 pt), and flavor (10 pt).

Sensory evaluations

Cell uniformity, cell size, cell wall thickness, grain, tenderness, softness, crumb color, and flavor of the MW/C and CON cakes were not significantly different. Neuzil and Baldwin (1962) also found that cakes baked conventionally or by microwaves only were similar in cell size, cell uniformity, texture and flavor, but the microwave cakes were less tender. However, Hill and Reagan (1982) found that yellow cakes baked by microwaves only scored lower in texture, appearance, tenderness, and flavor. Also, Martin and Tsen (1981) reported that cakes baked by microwaves only had a coarser texture, more irregular cells, and thicker cell walls than conventionally baked cakes.

The moisture, crust color and total sensory scores of single MW/C layers were lower than those of double MW/C or CON layers (Table 2). Although slight differences were found, these cakes were acceptable, and had better quality characteristics than those reported for cakes baked by microwaves only (Hill and Reagan, 1982; Neuzil and Baldwin, 1962; Street and Surratt, 1961). In contrast, double MW/C layers were rated equal or near-equal to CON layers. Thus, MW/C ovens offer a possible solution to faster (15–25%) baking and high quality cakes. However, more research is needed to evaluate other ovens and bakery products.

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A Research Note

Effects of Microwave/Convection Baking and Pan Characteristics on Cake Quality

C. T. STINSON

ABSTRACT

The quality of devil's food cake baked in a microwave/convection oven using different oven conditions and pan characteristics was assessed by sensory and objective methods. The most important factors were the number of layers baked at a given time and the baking pan characteristics, followed by initial oven temperatures. Crust color, moistness and cake symmetry were affected by these conditions. For example, single layers received a lower sensory score and had sticky and less red crusts than double layers. Cakes baked in glass-polyester microware pans were peaked, whereas those baked in aluminum pans were flat. Although slight differences in quality were found by both sensory and objective evaluations, all cakes were acceptable.

INTRODUCTION

MICROWAVE BAKING is faster than conventional means, but the resultant cakes are inferior, for they have less acceptable crust color, texture, surface contour and moistness (Hill and Reagan, 1982; Lorenz et al., 1973; Martin and Tsen, 1981; Neuzil and Baldwin, 1962; Proctor and Goldblith, 1948; Street and Surratt, 1961). The most undesirable characteristic is a light crust color. To remedy this, microwave ovens equipped with browning elements have been tried, but the cakes browned unevenly and cracked (Street and Surratt, 1961).

The recent availability of microwave/convection (MW/C) ovens offered a possible solution, since they are rapid and can brown bakery products. However, data are not available on the quality of cakes baked in these ovens.

In conventional baking, the size and shape of the baking pan, the baking pan material and the initial oven temperature affect the quality of layer cakes (Charley, 1950, 1952; Odland and Davis, 1982; Peet and Lowe, 1937). These factors also may be important in MW/C baking. Thus, the purpose of this research was to study the effects of both oven conditions and pan characteristics on the quality of devil's food cake baked in a MW/C oven.

MATERIALS AND METHODS

Cake preparation

A standard cake batter was prepared from a major brand of devil's food cake mix (Pillsbury Plus, Pillsbury Company, Minneapolis, MN), using common lots of all ingredients. Cake mix (525g), eggs (150g), oil (70g) and distilled water (300 mL) were mixed with a Sunbeam Mixmaster Mixer, Model 01080, at low speed for 30 sec, high speed for 2 min, and then 450g aliquots were poured into greased and floured 9-inch (22.9 cm) layer cake pans. All cakes were baked in a Sharp Carousel Household MW/C oven, Model R8320, operating with a frequency of 2450 MHz and with an output power of 650W (microwave) and 1500W (convection). The oven setting was "low mix," or 163°C (325°F) with 10% microwave power.

Cakes were baked using five different sets of oven conditions and pan characteristics (Tables 1 and 2). The independent variables included the number of layers baked at a given time, the initial oven temperature, the pan surface and the pan material.

Both aluminum and microware pans were evaluated since aluminum was suggested by the manufacturer of our specific MW/C oven. Usually aluminum pans are contraindicated for microwave ovens because they reflect microwave energy or cause arcing. However, they are acceptable for combination MW/C baking. Cake pans composed of different materials and surfaces were used, including shiny aluminum (Mirro Corporation, Manitowac, WI), dull aluminum (Mirro, Corporation, Manitowac, WI), and dull microware, which was composed of a glass and polyester compound with a xylan coating (Regal Ware, Inc., Kewaskum, WI).

The protocol for a daily experiment included baking a top and a bottom layer for each of the five sets of experimental conditions for a total of 10 layers. When two layers were baked simultaneously, they were positioned one above the other. The single layers were baked on the top or the bottom shelf.

The cooking times were dependent on the other baking conditions and pan characteristics. For example, more time was required for double than single layers because microwave cooking is very dependent on food quantity. Also more time was needed for the bottom than the top layers due to shielding. The endpoint cooking times were determined by sensory evaluations of layers cooked at various times for each set of conditions studied. The times which produced the highest quality cakes were used for this study.

The cooking times for the top and bottom layers were 14 min for the single layers, 20–22 min for the double layers baked in aluminum pans in a preheated oven, 19–21 min for the layers baked in a nonpreheated oven, and 20–26 min for the layers baked in microware pans. After baking, the cakes were placed on wire racks to cool for 15 min, removed from their pans, cooled for an additional 1.5 hr on wire racks and then placed in plastic storage bags.

The cakes were evaluated by a sensory taste panel within 2–3 hr after baking. The objective measurements of the cakes were made at standardized times within 24 hr after baking.

Objective measurements

Cake shape was characterized by the symmetry, volume, uniformity, and shrinkage indices according to Method 10-91 (AACC, 1983). The general method was modified so that an ink print rather than the cake itself was measured. Tenderness was measured objectively with a Precision Universal Penetrometer (Funk et al., 1969). The crust and crumb moisture contents were determined using Method 44-40 (AACC, 1983). Crust and crumb color was measured with a Colorgard system, model 2000/05 Colorimeter, using the Hunter color scale (Pacific Scientific, Silver Springs, MD). The instrument was calibrated with a standard white tile ("L" = 91.36, "a_L" = −1.03, "b_L" = 1.25).

Sensory evaluations

Five experienced panelists evaluated the cakes using a score card with quality characteristics and descriptive terms similar to Method 10-90 (AACC, 1983), except that crust color was added. The numerical scores were kept the same as outlined for each characteristic, and crust color was assigned a value of 10 to give a total score of 110. In addition, the descriptive terms for flavor, crumb color, and crust color were modified so that a score of 10 was excellent, 8 good, 6 fair, and 4 poor.

For each daily experiment, five coded 2 × 1 × 1 in pieces of cake were placed on white paper plates and randomly arranged on a separate tray for each panel member. Each tray contained samples from the same location in each layer, but all samples were taken from the center of each cake. The panelists evaluated the samples in individual

Author Stinson is affiliated with the Foods & Nutrition Program, Dept. of Health, Physical Education, & Recreation, Univ. of Louisville, Louisville, KY 40292.

Table 1—Moisture content, volume, symmetry, shrinkage, and uniformity indices, and penetrometer values of devil's food cake baked in a MW/C oven using different oven and pan conditions[z]

Experimental conditions	Moisture content (%) Crust	Moisture content (%) Crumb	Volume index (cm)	Symmetry index (cm)	Shrinkage index (cm)	Uniformity index (cm)	Penetrometer values (mm)
Single layers Preheated (163°C) oven Shiny aluminum pans	29.73 ± 0.25a	37.78 ± 0.24ab	11.92 ± 0.20a	−0.15 ± 0.09a	1.73 ± 0.05a	0.13 ± 0.02a	20.92 ± 0.93a
Double layers Preheated (163°C) oven Shiny aluminum pans	27.90 ± 0.34b	37.17 ± 0.52a	11.68 ± 0.20a	0.15 ± 0.08a	1.73 ± 0.09a	0.07 ± 0.03a	19.87 ± 0.93a
Double layers Nonpreheated (20-22°C) oven Shiny aluminum pans	28.13 ± 0.54b	37.70 ± 0.47ab	11.87 ± 0.11a	−0.02 ± 0.06a	1.77 ± 0.12a	0.10 ± 0.04a	20.68 ± 0.75a
Double layers Preheated (163°C) oven Dull aluminum pans	28.10 ± 0.52b	38.86 ± 0.14b	12.09 ± 0.13a	0.03 ± 0.07a	1.76 ± 0.08a	0.17 ± 0.04a	21.72 ± 0.23a
Double layers Preheated (163°C) oven Dull microware pans	27.83 ± 0.55b	38.95 ± 0.41b	12.22 ± 0.06a	1.01 ± 0.07b	1.57 ± 0.09a	0.13 ± 0.03a	19.37 ± 0.32a

[z] Values are expressed as mean ± standard error of the mean, n = 6. Means within columns followed by the same letter are not significantly different at the 5% level.

Table 2—Sensory scores and Hunter color values of devil's food cake baked in a MW/C oven using different oven and pan conditions[z]

Experimental conditions	Sensory scores[x]	Crust color[y] L	Crust color[y] a_L	Crust color[y] b_L	Crumb color[y] L	Crumb color[y] a_L	Crumb color[y] b_L
Single layers Preheated (163°C) oven Shiny aluminum pans	88.3 ± 0.4a	22.64 ± 0.70a	7.16 ± 0.20a	5.31 ± 0.24a	15.52 ± 0.22a	8.75 ± 0.39a	3.00 ± 0.28a
Double layers Preheated (163°C) oven Shiny aluminum pans	93.8 ± 0.4b	23.14 ± 0.39a	8.05 ± 0.04b	5.75 ± 0.22a	15.22 ± 0.16a	9.14 ± 0.29a	2.76 ± 0.11a
Double layers Non-preheated (20-22°C) oven Shiny aluminum pans	93.9 ± 0.3b	23.72 ± 0.34a	7.83 ± 0.11b	5.73 ± 0.17a	14.99 ± 0.11a	9.11 ± 0.60a	2.94 ± 0.19a
Double layers Preheated (163°C) oven Dull aluminum pans	92.1 ± 0.5b	24.13 ± 0.18a	7.80 ± 0.11b	5.96 ± 0.05a	16.14 ± 0.06a	8.73 ± 0.28a	2.93 ± 0.29a
Double layers Preheated (163°C) oven Dull microware pans	92.5 ± 0.3b	21.79 ± 0.84a	8.44 ± 0.26b	5.63 ± 0.17a	15.51 ± 0.68a	9.13 ± 0.32a	2.88 ± 0.15a

[x] Total score is 110, based on cell uniformity (10 pt), cell size (10 pt), cell wall thickness (10 pt), grain (16 pt), moistness (10 pt), tenderness (14 pt), softness (10 pt), crumb color (10 pt), crust color (10 pt), and flavor (10 pt).

[y] Color values: L = lightness, 100 = white, 0 = black; a_L = redness; b_L = yellowness.

[z] Values are expressed as mean ± standard error of the mean, n = 6. Means within columns followed by the same letter are not significantly different at the 5% level.

sensory booths with overhead fluorescent lighting. Cool (20–22°C) water was provided for the panel to rinse between samples.

The sensory scores and objective measurements of the top and bottom layers for each set of conditions were averaged, since preliminary analyses indicated no differences between the two layers. These data were analyzed further using analysis of variance, followed by the "Q" test (Snedecor and Cochran, 1980).

RESULTS & DISCUSSION

Moisture

Separate moisture measurements were made for the crust or crumb alone because sticky crusts is a common microwave problem and thus may be important in MW/C baking. The moisture of crusts of single layers was 29.7% compared to 27.8–28.1% for double layers (Table 1). This 1.5–2% difference resulted in sticky, less desirable crusts. The higher moisture of the single crusts may be due to shorter cooking times (5–7 min) and thus less exposure to dry, convective heat. In earlier experiments, the single crusts were improved by longer cooking times (30–60 sec), but their crumb also became drier (36%) and unacceptable.

The crumb moisture of cakes baked in shiny aluminum pans was the same whether baked as single or double layers, and in a preheated or nonpreheated oven. However, there were pan surface differences; cakes baked in dull aluminum or dull microware pans were 1–2 percentage points higher in moisture than those baked in shiny aluminum pans, and as double layers in a preheated oven.

Volume, symmetry, shrinkage, and uniformity indices

Ideally, high quality cakes have slightly rounded, symmetrical tops, a large volume, and a low degree of shrinkage. The symmetry index describes the top surface of layer cakes, which may be sunken, flat or rounded and indicated by negative, zero or positive values, respectively. The uniformity index of a high quality cake is zero, which indicates a cake with equal halves. In contrast, a positive value indicates a lop-sided cake.

Cakes baked in microware pans had a greater symmetry index than those baked in shiny or dull aluminum pans (Table 1). Also, these cakes had peaked tops compared to cakes baked in aluminum pans which were flat. In part, cake symmetry was due to pan depth for the microware pans were 5.1 cm deep, whereas the aluminum pans were 3.8 cm deep. Similar differences in cake shape due to pan depth have been reported for conventional baking (Charley, 1952).

The symmetry indices of all cakes baked in aluminum pans were the same regardless of surface. However, cake symmetry was affected by the number of layers baked at a given time and the initial oven temperature. For example, both single layers, and layers baked in a nonpreheated oven had negative index values (−0.02 to −0.15 cm), which indicated slightly sunken cakes with an undesirable appearance. In contrast, the

double layers baked in a preheated oven had positive index values and were flat.

The volume, shrinkage or uniformity indices were not affected by the different conditions of this study. On the other hand, conventionally baked cakes had more volume and were flatter when baked in dull versus shiny aluminum pans (Charley, 1950).

Penetrometer values

The penetrometer values of the cakes were not significantly different (Table 1). This was confirmed by the panel, who found no differences in cake tenderness.

Color

The single layers had a lower "a_L" (redness) score for crust color than the double layers, but no differences were found in the "b_L" (yellowness) or "L" (lightness) scores (Table 2). Even though the differences in "a_L" scores were small, the panel rated the crust color of the single layers lower. The crumb color of the cakes was not affected by the conditions used in this study.

Sensory evaluations

The single layers baked in shiny aluminum pans in a 163°C preheated oven received a lower sensory score than the double layers baked using any of the conditions studied (Table 2). The characteristics which contributed to this lower score were moistness and crust color.

The variable in the baking conditions which had the most effect on sensory quality was the numbers of layers baked at a given time. The initial oven temperature and the pan characteristics were less important. Street and Surratt (1961) also found that the size and shape of the baking pan had little effect on the palatability qualities of yellow cakes baked in a microwave oven.

Sensory scores for the top versus the bottom layers for each set of conditions were also compared, but no differences were found. Thus, pan location did not affect the sensory quality of cakes baked using the conditions of this study.

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GREENING OF GARLIC PUREE. . .From page 1577

tensified at low temperature and decreased with warm storage. Similar experiments, using a commercially grown Creole garlic cultivar and several lots of home grown bulbs of unknown cultivar, showed the same response to storage temperature. These experiments were done over a period of 3 years. Bulbs were switched from greening to nongreening and back again several times by moving them from one storage temperature to another. In one case this was done three times. Each time it took longer to make the change.

The neutral amino acids were investigated since they are involved in both the pinking of onions and the greening of garlic (Lukes, 1958; Shannon, 1961). Greening and nongreening bulbs were analyzed and the outstanding difference between the two was the reduced amount of PECSO in the nongreening type, e.g., 0.1–0.3 mg/g of garlic in nongreening and as high as 1.62 mg/g in the greening type. This amino acid is the precursor of the onion lachrymator (Virtanen and Spare, 1961) and its presence in garlic is not well known. The amount of PECSO decreased in warm storage and increased in cold storage. The central role of PECSO in the greening reaction was demonstrated by adding small amounts of it to nongreening, nonheated puree. The mixture turned a depth of green which depended upon the amount of PECSO added (Fig. 1). The equation for the line of best fit, between 0.1 and 1.8 mg PECSO, was absorbance = (0.59 + 0.67) × mg PECSO. The correlation was 0.985 and was significant at the 99% level. The puree that had no PECSO added was pale yellow. The color with 0.1 mg PECSO/g garlic was very light green; 0.4 was green and 1.0 was dark bluish-green.

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A Research Note

Characterization and Distribution of Aerobic, Spore-Forming Bacteria from Cacao Fermentations in Bahia

ROSANE FREITAS SCHWAN, MARIA CRISTINA D. VANETTI, DAISON OLZANY SILVA, ALEX LOPEZ, AND CÉLIA A. DE MORAES

ABSTRACT

Aerobic spore forming bacteria isolated from traditional cacao fermentations in Bahia were identified in the genus *Bacillus: B. subtilis, B. licheniformis, B. firmus, B. coagulans, B. pumilus, B. macerans, B. polymyxa, B. laterosporus, B. stearothermophilus, B. circulans, B. pasteurii, B. megaterium, B. brevis*, and *B. cereus*. In the first 8 hr of the fermentations, similar percentages of ten species were found in the distribution. During the fermentation process the amount of spore-forming bacteria increased and *B. subtilis, B. circulans*, and *B. licheniformis* appeared more frequently.

INTRODUCTION

CACAO BEANS used in the manufacture of chocolate must undergo an on-the-farm fermentation and drying process during which the precursors of chocolate flavor are formed. During this fermentation process, various microorganisms develop in the pulp surrounding the bean producing substances, mainly alcohols and acids, which affect the curing and the flavor properties (Forsyth and Rombouts, 1951; Camargo et al, 1963; Maravalhas, 1966; 1972; Ostovar and Keeney, 1973). These acids and other microbial by-products, when present in moderate quantities, contribute positively towards the flavor of chocolate (Lopez, 1974); however, in high concentrations, these may result in disagreeable off-flavors which render the product useless for the manufacture of good quality chocolate.

Microorganisms active during the fermentation of cacao develop in a succession of yeasts, lactic and acetic acid bacteria, and spore forming bacteria (Roelofsen, 1958). Studies confirmed the presence of spore forming bacteria during fermentation but no attempt was made to identify them (Knapp, 1937; Bridgland and Friend, 1957; Roelofsen, 1958; Forsyth and Quesnel, 1963). These dominate the fermentation by the fourth day, by which time, most of the flavor precursors have already been formed. These bacteria may still influence the flavor of the product as they appear at that stage when off-flavors are generally encountered.

The purpose of the study was to identify and characterize the aerobic spore forming bacteria present in cacao beans during the traditional Bahia fermentations.

MATERIAL & METHODS

Sampling procedures

Cacao bean of the COMUN hybrids from the fields of the cacao Research Center, CEPLAC at Ilhéus, Bahia were fermented in the traditional Bahia method (Passos et al., 1984).

The first sample consisting of 20 beans (Passos *et al* 1984) was taken as the sweat boxes were being filled and the second 8 hr later. The remainder of the samples were taken at 12-hr intervals for 13 intervals, a total fermentation time of 164 hr. Samples were taken from locations 30 cm from the walls and 45 cm below the surface.

Isolation details

Extracts were prepared by agitating 20 beans in an Erlenmeyer flask containing 200 mL Ringer solution (Merck) and 5g sterilized fine sand for 20 min with a magnetic stirrer. The extracts were heated to 80°C for 20 min to destroy vegetative bacteria. Decimal dilutions were plated on medium recommended by Ostovar and Keeney (1973) with 1% cacao pulp added, and, after incubation at 37°C for 48 hr, isolations were made by collecting a number of colonies equivalent to the square root of the total count (FDA, 1972).

The isolated aerobic spore-forming bacteria were preserved at 5°C in screw-capped tubes containing sterile soil in order to maintain their natural characteristics (Martin, 1964), and on nutrient agar slants at 5°C with periodic transfers.

Identification of the isolates

The morphological characteristics of the vegetative and spore forms of the bacteria were determined by Gram staining. Species were identified by the following characteristics: carbohydrate fermentation (glucose, manitol, xylose, and arabinose) (Gibson and Gordon, 1974); catalase activity; nitrate reduction (Harrigan and McCance, 1976); acetoin formation (Norris et al., 1981); starch, gelatin and casein hydrolysis (Harrigan and McCance, 1976); citrate utilization; acid production in litmus milk; phenylamine deamination (Harrigan and McCance, 1976); anaerobic growth (Norris et al., 1981; Costilow, 1981); thermotolerance at 50° and 65°C (Norris et al., 1981); growth in 7% saline and motility (Norris et al., 1981).

RESULTS & DISCUSSION

ONE HUNDRED FIFTEEN CULTURES of aerobic spore forming bacteria were isolated from the samples of cacao beans taken during the fermentation process. These were identified as: *Bacillus subtilis, B. licheniformis, B. coagulans, B. pumilus, B. macerans, B. laterosporus, B. pasteurii, B. megaterium, B. stearothermophilus, B. circulans, B. firmus, B. brevis, B. polymyxa*, and *B. cereus*. With the exception of *B. brevis*, all others were found in cacao fermentations in Trinidad (Ostovar and Keeney, 1973). Some species, such as *B. subtilis, B. licheniformis*, and *B. circulans*, were encountered more frequently during the fermentation process than others (Fig. 1). The percentage distribution of *Bacillus* species during fermentation (Fig. 1) suggests an initial diverse contamination of the beans. The number of aerobic spore-forming bacteria remained virtually unchanged during the early part of fermentation which indicates that the conditions that prevailed in the fermenting mass at this stage did not favor their growth. After 120 hr the percent aerobic spore-forming bacteria increased progressively until the end of fermentation. This change in sequence coincided with increases in O_2 tension, pH and the temperature (> 50°C) of the fermenting mass (Schwan, 1984).

The presence of *Bacillus* in all samples taken during fermentation does not signify that conditions were favorable for their development. The ability of these microorganisms to form spores and survive adverse growth conditions may explain their dominance over other groups of microorganisms during the later high temperature phase observed in the traditional Bahia

Authors Schwan and Lopez are with Divisão de Tecnologia e Engenharía Agricolas, Centro de Pequisas do Cacau, CEPLAC, Caixa Postal 7 - Itabuna, Bahia - 45.600-Brazil. Authors Vanetti, Silva, and de Moreau are with the Departamento de Biologia Geral, Universidade Federal de Viçosa, MG - 36.570, Brazil.

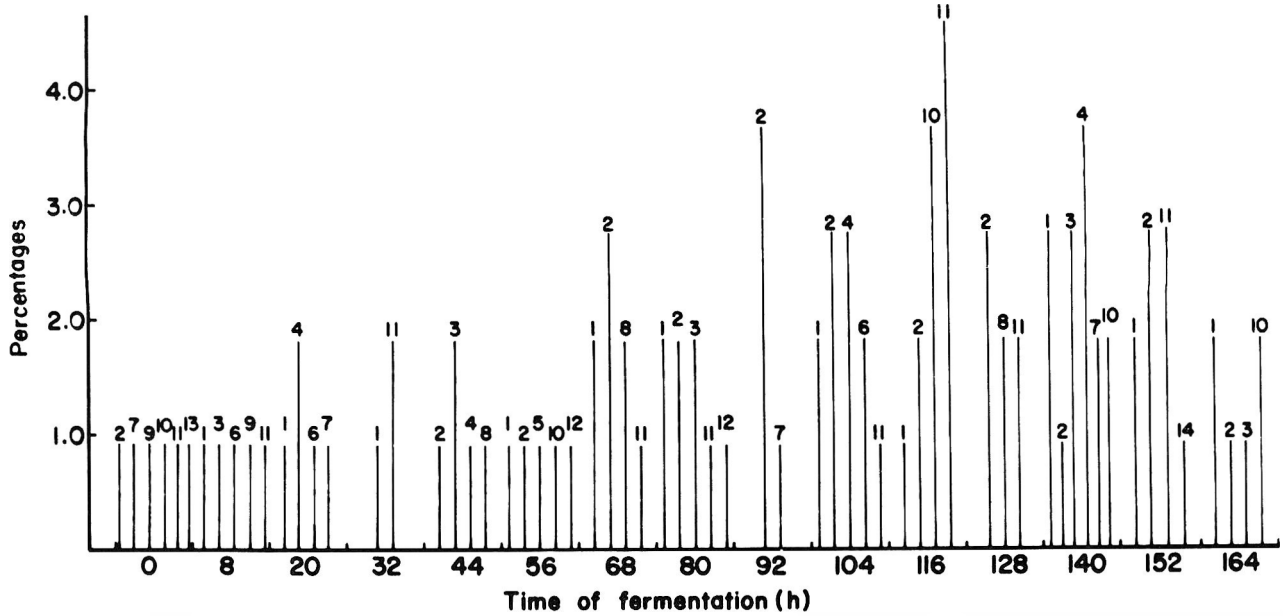

Fig. 1—Percent distribution of 110 isolates of 14 species of genus Bacillus present in traditional fermentations of cocoa beans in Bahia: *B. subtilis* (1), *B. licheniformis* (2), *B. coagulans* (3), *B. pumilus* (4), *B. macerans* (5), *B. polymyxa* (6), *B. firmus* (7), *B. laterosporus* (8), *B. cereus* (9), *B. stearothermophilus* (10), *B. circulans* (11) *B. pasteurii* (12), *B. megaterium* (13), and B. brevis (14).

fermentation (Schwan, 1984). In fact, Barrile et al. (1971), demonstrated the tolerance of *B. stearothermophilus*, *B. coagulans*, and *B. circulans* to high temperatures by isolating them from cacao beans that had been subjected to drying and roasting temperatures.

The aerobic spore-forming bacteria are able to produce a variety of chemical compounds relative to fermentation conditions. It is quite likely that their metabolic products contribute to the acidity and off-flavors of cured cacao beans. It has been suggested that the C_3–C_5 free fatty acids that are found during the latter aerobic phase of fermentation are produced by some *Bacillus* species like *B. subtilis*, *B. cereus*, and *B. megaterium* and may be responsible for off-flavor of chocolate (Lopez and Quesnel, 1973). Other organic compounds such as acetic and lactic acids, 2,3-butanediol and tetramethylpyrazine which influence the flavor of chocolate are also produced by *Bacillus* species (Buchanan and Gibbons, 1974; Lopez and Quesnel, 1971 and Zak et al., 1972). As the aerobic spore-forming bacteria in the final stages of the fermentation process predominate over other microorganisms, it could be possible that they contribute to the off-flavors in fermented cacao. Thus, an understanding of the metabolism of this group of microorganisms in relation to the fermentation process in conjunction with analytical studies may lead to a clear understanding of the factors related to the development of foreign flavors which appear in Brazilian fermented cacao.

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A Research Note

Aerobic Incubation of *Clostridium perfringens* in the Presence of an Oxygen-Reducing Membrane Fraction

C. B. HOSKINS, E. RICO-MUNOZ, and P. M. DAVIDSON

ABSTRACT

The recovery and growth rates of *C. perfringens* were evaluated in media to which an oxygen-reducing membrane fraction from *E. coli* was added. Each of three *C. perfringens* strains was incubated in Tryptose Sulfite Cycloserine agar, with and without the membrane fraction. Recoveries were greater on media incubated aerobically with the membrane fraction than on the other formulations incubated anaerobically or aerobically. *C. perfringens* ATCC 12917 reached stationary phase more rapidly in fluid thioglycollate medium (FTM) plus the membrane fraction than in FTM alone. Results showed that the membrane fraction allowed aerobic incubation for the recovery of *C. perfringens*.

INTRODUCTION

CLOSTRIDIUM PERFRINGENS is one of the most common causes of foodborne illness. Laboratory culturing of *C. perfringens* does not require strict anaerobic conditions if a liquid medium with a reducing agent such as thioglycollate is used. However, when using solid media for isolation and enumeration of *C. perfringens* from foods, anaerobic incubation is required (Harmon and Duncan, 1984). The solid medium of choice for *C. perfringens* is currently Tryptose Sulfite Cycloserine (TSC) agar (Barach et al., 1974; Hauschild and Hilsheimer, 1974; Mead, 1985).

Adler and co-workers were the first to demonstrate that an oxygen-reducing membrane fraction isolated from *Escherichia coli* allowed the growth of selected *Clostridium* species in an aerobic environment (Adler and Crow, 1981; Adler et al., 1981, 1983). If their material could be successfully applied to media normally used in the isolation of *C. perfringens*, the detection of this microorganism in foods could be greatly facilitated.

The objective of this study was to determine the effect of the oxygen-reducing membrane fraction, termed P_2, on the recovery and growth rate of *C. perfringens* under aerobic conditions.

MATERIALS & METHODS

Bacterial strains

Individual strains of *C. perfringens* (ATCC 12917, NCTC 10239 and NCTC 8798) were maintained in FTM at 4°C. Prior to testing, each strain was transferred to fresh FTM and incubated at 42°C for 18 hr.

Membrane fraction

The membrane fraction (P_2) was obtained in the frozen state from the Biology Division, Oak Ridge National Laboratories, Oak Ridge, TN and stored at −20°C. The isolation technique for P_2 was described previously (Adler et al., 1981). When used, P_2 was added immediately prior to pouring the agar (temperature: 45–50°C) at a rate of 20 μL/mL medium.

The authors are affiliated with the Dept. Food Technology & Science, Univ. of Tennessee, P.O. Box 1071, Knoxville, TN 37901-1071.

Table 1—Enumeration of Clostridium perfringens *strains in media with and without an oxygen-reducing membrane fraction*

Medium[a]	Overlay	Growth condition	Colony forming units/mL (× 10^5) ATCC 12917	NCTC 10239	NCTC 8798
TEST 1[b]					
TSC	yes	Aerobic	10	10	25
TSC	yes	Anaerobic	56	21	106
TSC	no	Aerobic	<1	<1	<1
TSC[c]	no	Anaerobic	52	26	101
TSC-sb + P_2	yes	Aerobic	244	229	299
TSC-sb + P_2	no	Aerobic	220	258	279
TSC-sb	yes	Aerobic	66	30	82
TSC-sb	yes	Anaerobic	79	63	144
TSC-sb	no	Aerobic	18	<1	<1
TSC-sb[d]	no	Anaerobic	46	52	129
TEST 2[e]					
TSC	yes	Anaerobic	700	580	210
TSC[c]	no	Anaerobic	620	580	220
TSC + P_2	yes	Aerobic	1160	1220	370
TSC + P_2	no	Aerobic	790	600	260

[a] TSC = Tryptose Sulfite Cycloserine; sb = sodium bisulfite; P_2 = membrane fraction.
[b] Numbers shown are the mean of two replications.
[c] TSC control.
[d] TSC-sb control.
[e] Numbers shown are the mean of three replications.

Media

The solid medium used was TSC without egg yolk enrichment. It, along with FTM, was prepared as described by Harmon and Duncan (1984). Another batch of TSC was prepared without the reducing agent sodium bisulfite (TSC-sb). The media were sterilized at 121°C for 15 min. Dissolved oxygen was measured with a Chemical Microsensor System (Transidine General Corporation, Ann Arbor, MI) and a miniature Clark-style electrode. The electrode was placed in an oxygen uptake chamber containing 1 ml medium initially equilibrated with air at 23–25°C.

Recovery rate

The organisms were subcultured in FTM for 18 hr at 42°C, diluted in 0.1% peptone and plated in each of the following media: TSC, TSC-sb, TSC-sb + P_2 and TSC + P_2. One-half of the plates were overlayed with the same medium. One-half of the plates without P_2 were incubated anaerobically using the Gas-Pak Anaerobic System (BBL, Cockeysville, MD); the remainder were incubated aerobically. Plates with media containing P_2 were incubated aerobically. All plates were incubated at 42°C for 48 hr.

Growth rate

To evaluate the effect of P_2 on growth rate, *C. perfringens* ATCC 12917 was incubated at 42°C for 18 hr in FTM. Then 0.5 mL culture was added to each of 24 sterile tubes which contained either 5 mL FTM or 5 mL FTM plus P_2. All tubes were placed in a 42°C incubator and one tube from each set was removed every 30 min. After thorough agitation, turbidity was determined spectrophotometrically (Spectronic 20, Bausch and Lomb, Rochester, NY).

Statistical analysis

All experiments were replicated at least twice. Data were analyzed using Analysis of Variance and, if significant differences were de-

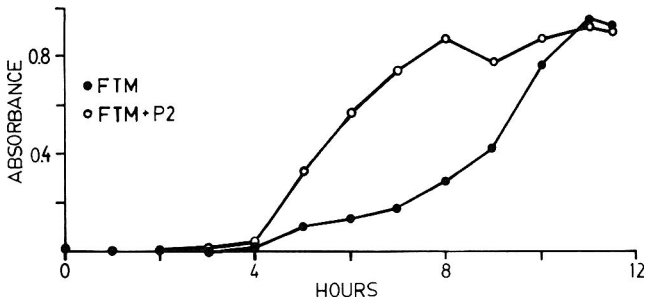

Fig. 1—Rate of growth of Clostridium perfringens *ATCC 12917 in fluid thioglycollate medium with (FTM + P_2) and without (FTM) an oxygen-reducing membrane fraction.*

tected at $P<0.05$, the means were separated using Duncan's Multiple Range Test.

RESULTS & DISCUSSION

IN THE TSC-sb medium, no reduction in oxygen was noted indicating that sodium bisulfite was the only reducing agent in TSC. However, once P_2 was added to TSC-sb, the dissolved oxygen decreased to zero within approximately 2 min.

The effect of P_2 on the recovery of *C. perfringens* in TSC is shown in Table 1. In test 1, TSC and TSC-sb plates incubated anaerobically and having no overlay served as the controls. The greatest recovery ($P<0.05$) of *C. perfringens* was achieved when P_2 was added to TSC medium without sodium bisulfite incubated aerobically. There was no significant difference ($p>0.05$) in counts on the media containing P_2 with or without an overlay, therefore they were averaged for the following comparisons. The recovery of strain ATCC 12917 cultured in TSC-sb + P_2 was 446% of the TSC control and 504% of the TSC-sb control. NCTC 10239 cultured in TSC-sb + P_2 was recovered at 936% of the TSC control and 468% of the TSC-sb control while NCTC 8798 cultured in P_2 was recovered at 286% and 224% of the TSC and TSC-sb controls, respectively.

In test 2, a comparison was made between the recovery of *C. perfringens* on complete TSC with and without P_2 (Table 1). The percentage increase in recovery of the microorganism on TSC + P_2 compared to the TSC control was reduced from that found in test 1. This reduction was most likely due to the presence of the sodium bisulfite. The overlayed TSC + P_2 plates incubated aerobically still had a significantly greater ($p<0.05$) number of colonies than the TSC control (no overlay, anaerobic incubation). No difference was detected between the TSC + P_2 without overlay and the TSC treatments incubated anaerobically.

The growth curves of *C. perfringens* ATCC 12917 in FTM with and without P_2 are shown in Fig. 1. Stationary phase of strain ATCC 12917 was reached in approximately 8 hr in FTM + P_2 while over 11 hours were required in FTM without P_2. Turbidity of the culture in FTM was approximately 30% of maximum at the same time maximum turbidity in FTM + P_2 was achieved.

The significance of the above findings are multiple. First, anaerobic equipment and precautions were found to be unnecessary to grow the organisms. Secondly, aerobic incubation allowed the plates to be monitored periodically without interrupting anaerobic conditions. Thirdly, the number of viable *C. perfringens* recovered was significantly increased when P_2 was added to the media. Finally, the growth rates of the organisms were improved by P_2 making it possible to speed up laboratory culturing procedures.

Because of its convenience and ease of use, this new technique might be used in the future to isolate, identify and rapidly culture many types of anaerobes from food products.

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The Institute of Food Technologists

☐ The Institute of Food Technologists is an educational and scientific society of food professionals—technologists, scientists, engineers, educators, and executives—in the field of food technology. Food technologists apply science and engineering to the research, production, processing, packaging, distribution, preparation, evaluation, and utilization of foods. Individuals who are qualified by education, special training, or experience are extended an invitation to join in professional association with the select group of the food industry's scientific and technological personnel who are IFT members. Membership is worth many times its modest cost, reflecting positive benefits, stimulation, and opportunities for the individual in his or her business or profession.

Objectives

IFT has several major aims: to stimulate investigations into technological food problems; to present, discuss, and publish the results of such investigations; to raise the educational standards of food technologists; and to promote recognition of the scientific approach to food and the basic role of the food technologist in industry. All of these activities have the ultimate objective to provide the best possible foods for mankind.

Organization and Progress

Organized July 1, 1939, at Cambridge, Mass., with a membership of less than 100, the Institute has grown to nearly 23,000. It is worldwide in scope with more than 3,000 of its membership overseas.

Qualifications for Membership

Professional Members. Any person who meets the following minimum requirements: (1) Bachelor's degree or higher from a college or university with a major in one or more of the sciences or branches of engineering associated with food technology; and (2) Five years of professional experience in food technology, for which a master's degree may be presented as the equivalent of one year's experience; a doctor's degree, the equivalent to three year's experience.

Members. Any person active in any aspect of the food industry and who evidences interest in supporting the objectives of IFT. Recent graduates who are completing their experience requirement for Professional Member status.

Student Members. Any person who is registered as a full-time student in an educational institution and who is pursuing candidacy for an associate degree or higher in one or more of the sciences or branches of engineering associated with food technology shall be eligible for membership as a Student Member. After graduation, a student may convert automatically to Member without obligation for the higher Member dues until the following calendar year.

Dues

Professional Members and Members—$55 a year; includes subscription to *Food Technology;* option to subscribe to the *Journal of Food Science* at members' special rate of $15.
Student Members—$15 a year; includes subscription to one IFT journal, automatic membership in the Student Association, and option to subscribe to the other journal at $7.50.
Emeritus Members—no dues; includes option to subscribe to either or both journals at $7.50 each.

Publications

The Institute publishes two journals. *Food Technology,* issued monthly, is the official journal of the Institute; *Journal of Food Science,* issued bimonthly, is devoted to basic and applied research papers of fundamental food components and processes.

Regional Sections

Where 25 or more members live within commuting distance of a given geographic area, a regional section may be established. Presently, there are 50 regional sections.

Divisions

Where 50 or more members of the Institute have a common interest in a particular broad-based discipline of food technology, they may form a division. There are presently 12 divisions serving the areas of Biotechnology, Carbohydrates, Food Engineering, Food Packaging, Foodservice, Food Microbiology, Muscle Foods, Nutrition, Quality Assurance, Refrigerated and Frozen Foods, Sensory Evaluation, and Toxicology and Safety Evaluation.

Specialized technology groups (STGs)

When 25 or more members have a common interest in a rather narrow, product-oriented or similar special area, they may form a technology group to serve the needs of this specialized area. There are currently five STGs—Citrus Products, Dairy Products, Extension, Fruit and Vegetable Products, and Seafood Products.

Student Association

All Student Members of IFT are automatically members of the Student Association, which provides special services and activities for students. This Association, which is run by and for the students, also provides the organizational mechanism for giving students a voice in IFT affairs.

Student Chapters

An IFT Student Chapter certificate may be granted to a group of students enrolled in the food science and technology curriculum in a particular school who have organized to form a student club. There are 38 student chapters.

Affiliate Organizations

Affiliate certificates may be granted to food technology organizations outside the U.S.A. There are currently 16 chartered Affiliate Organizations.

Annual Meetings

An Annual Meeting of the Institute provides a specially organized technical program, awards program, and an exposition (FOOD EXPO) of equipment, services, processes, and ingredients. The program is designed to emphasize current trends and technological developments.

Awards

The Institute presents the following awards, usually annually:

Nicholas Appert Award. Purpose of this award (medal furnished by the Chicago Section, and $5,000 by IFT) is to honor a person for pre-eminence in the contributions to the field of food technology.

Babcock-Hart Award. Purpose of this award ($3,000 furnished by the Nutrition Foundation and a plaque by IFT) is to honor a person for contributions to food technology that have improved public health through some aspects of nutrition or more nutrious food.

IFT International Award. Purpose of this award (plaque and $3,000 furnished by IFT) is to recognize an IFT Member for promoting international exchange of ideas in food technology.

IFT Food Technology Industrial Achievement Award. Purpose of this award (plaque to company or companies involved) is to recognize and honor the developers of an outstanding new food process and/or product representing a significant advance in the application of food technology to food production, successfully applied in actual commercial operation.

Wm. V. Cruess Award for Excellence in Teaching. Purpose of this award (medal furnished by the Northern California Section and $3,000 by IFT) is to recognize excellence in university teaching in food science and technology.

Samuel Cate Prescott Award for Research. Purpose of this award ($3,000 and a plaque furnished by IFT) is to recognize a research scientists 36 years of age or younger who has demonstrated outstanding ability in food science or technology.

Fellows Awards. Any Professional Member who has been active for at least 15 years and who has outstanding contributions to the field of food science and technology is eligible to be elected a Fellow of the Institute.

Carl R. Fellers Award. Recognizes individual members of IFT and Phi Tau Sigma who have served and brought honor and recognition to the profession of food science and technology. Winner receives $1,000 from Phi Tau Sigma and a plaque from IFT.

Scholarship/Fellowship Program

To attract and encourage students in the field of food science and food technology, the Scholarship/Fellowship program is offered to worthy and deserving students, primarily on the basis of scholastic ability.

IFT sponsors ten $500 Freshman and ten $750 Sophomore awards.

In addition, eight Freshmen, two Sophomore, and 52 Junior/Senior as well as 23 Graduate Fellowships, ranging in amount from $500 to $10,000 annually, are sponsored by various food companies and IFT and administered by IFT. Details are available in the booklet. *IFT Administered Fellowship/Scholarship Program*—1987–1988, available from IFT's Scholarship Department.

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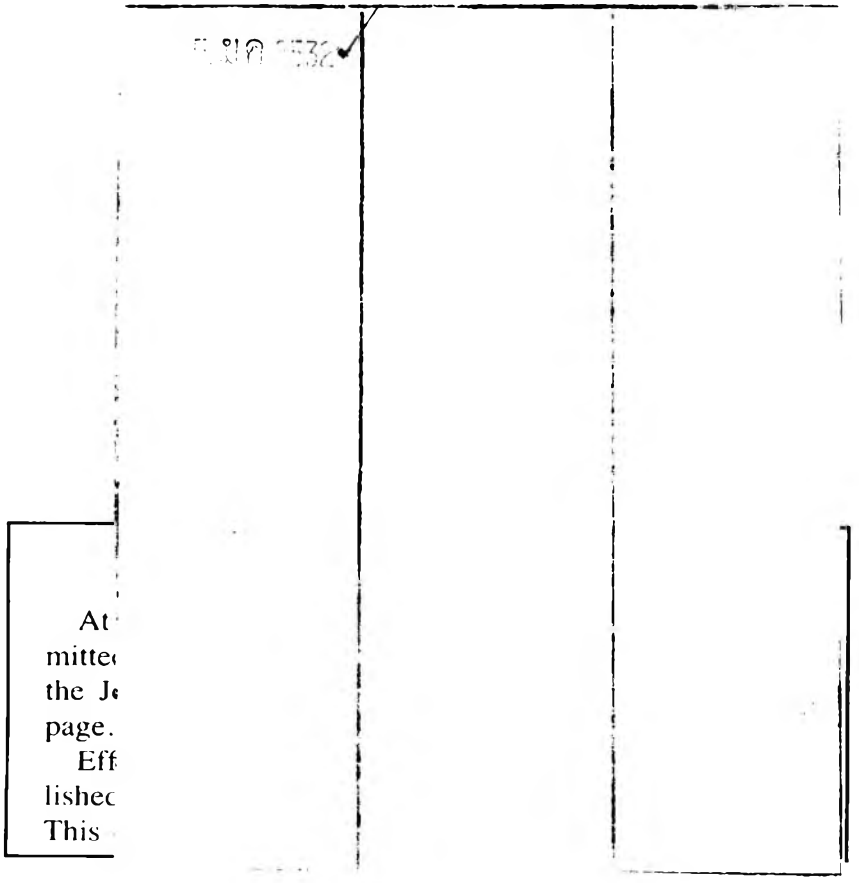

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