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Volume 55, No. 6

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JOURNAL OF FOOD SCIENCE

Basic Research, Applied Science & Engineering

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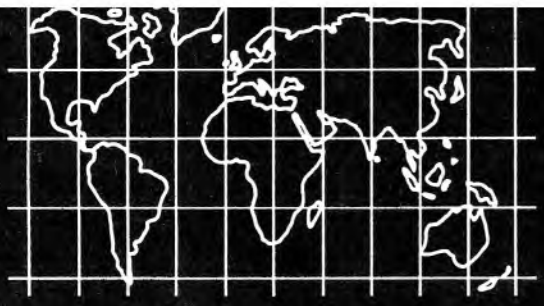
Volume 55: Number 6



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JOURNAL OF FOOD SCIENCE

6 Nov.-Dec. 1990
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MEMO *FROM THE SCIENTIFIC EDITOR*

This year JFS has undergone considerable change in organization, operating policy and procedures. I will forego the usual statistical summary of manuscripts submitted, rejected and accepted during the year. Suffice to say we had more manuscripts submitted this year than the previous year, and the rejection rate has increased to about 48%. At this point it is more important to describe some changes that have taken place or will be initiated in the near future. We began the year with a new organization of eight Associate Scientific Editors representing a broad cross-section of disciplines and commodities from the membership of IFT. They, along with some changes in policies regarding style and review procedures, are introduced to you in the December, 1990 issue of Food Technology. If you submitted manuscripts during 1990, you've already met one or more of them, through correspondence or phone calls, if not in person.

At the Fall 1989 Executive Committee meeting the new Editor's Board was given the charge to continue to upgrade scientific quality and merit of manuscripts accepted for publication; expanding, building and improving on the excellent foundation provided by the former Scientific Editor and his Associates. Thus, the Editorial Board decided to be more conservative in judging scientific merit and especially appropriateness of submitted manuscripts. This, more than any other reason, resulted from feedback we were receiving from you, our readers, and contributors. Hence, on manuscripts that are somewhat questionable in scientific merit or appropriateness of subject/scientific approach/design, we are now tending to favor rejection, after careful consideration among Editors.

We have initiated new policies regarding style, mainly to make our publications easier to comprehend, more precise and concise. If you have not done so already, may we suggest you read carefully a copy of the most recent Style Guide [JFS 55(2): 597 (1990)] or write us and we will provide a copy. Because of many complaints from reviewers about the difficulties of reviewing manuscripts not in the proper style and format, we have become rather demanding and particular about that subject. Save yourself some time by studying the new Style Guide before preparing manuscripts.

We are soon to initiate some new methods of communicating with you, our contributors and readers. Beginning in Vol. 56 and throughout 1991, each issue will contain a memo from me or a member of the Board of Editors. These will cover a range of subjects from policies, guidelines and observations on manuscripts received, through helpful hints to authors. Also, plan to stay the final afternoon of the IFT Annual Meeting and attend our Forum on "How to Get Your Paper Published in JFS." It will concern suggestions for improving submitted manuscripts and describe editors/reviewers pet peeves. There will be something for everyone and you who use JFS will find it well worthwhile.

This first year initiating a new system has been somewhat hectic and, to say the least,—"interesting!" In the rush to overcome the backlog of manuscripts which resulted from transferring editorial systems we've had a few traumatic moments. The system is well established and functional now, and as a result, you will see continually improving scientific quality in this Journal. We appreciate your contributions and are open to any suggestions/criticisms you wish to convey. We appreciate your cooperation and understanding during the transition and look forward to working with you through the coming year.

As you can discern, we are doing what we can to make it easier for reviewers to direct undivided attention to the scientific merit of manuscripts and not be diverted by mechanical/grammatical problems of format and style. This can help us upgrade scientific quality. The critical influence on our published quality is the conscientiousness and professionalism of the reviewers. You have done a good job in the past year and we thank you for your time and efforts. Following is a list of those who have assisted in this respect in 1990. We apologize for others we may have missed. We trust we can continue to depend on you for your scientific and professional support.

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Effects of Feeding Elevated Levels of Monounsaturated Fats to Growing-finishing Swine on Acceptability of Boneless Hams

S. D. SHACKELFORD, J. O. REAGAN, K. D. HAYDON, and M. F. MILLER

ABSTRACT

The processing and sensory characteristics were investigated on boneless, fully-cooked hams produced from swine fed diets high in monunsaturated fat. First impression and overall tenderness scores were higher for hams from the animal fat, safflower oil and sunflower oil treatments than from the control and canola oil treatments ($P < 0.05$). No differences were noted across treatments for juiciness, saltiness, flavor intensity or overall palatability; however, off-flavors were ($P < 0.05$) most frequently in samples from the canola oil treatment. Hams from swine fed 10% safflower oil or sunflower oil were acceptable in sensory, physical and chemical characteristics.

INTRODUCTION

SATURATED FAT continues to be implicated as a principal cause of elevated plasma cholesterol (Mattson and Grundy, 1985). Consumption of saturated fatty acids increased plasma LDL-cholesterol in man (Mattson and Grundy, 1985) which has correlated with coronary heart disease. Consequently, reduction of saturated fat consumption has developed as a chief diet and health issue in regard to meat consumption.

Grundy (1986) reported diets containing elevated levels of high oleate safflower oil lowered plasma cholesterol in man. Monogastric animals are very susceptible to tissue fatty acid alteration through dietary modification (Villegas et al., 1973; Skelley et al., 1975; St. John et al., 1987). Increasing the consumer acceptability of fully-cooked, boneless hams is of economic importance to the swine industry as cured hams are a major commodity, account accounting for 47% of total cured pork production (AMI, 1988). The objectives of our study were to determine the effects of feeding high oleate diets to swine on the palatability, processing yields and physical attributes of boneless hams made from their meat.

MATERIALS & METHODS

SIXTY BARROWS and gilts were given one of five dietary treatments consisting of a control diet of corn and soybean meal and four similar test diets that contained a 10% addition of either animal fat, safflower oil, sunflower oil or canola oil. The treatments were initiated when animals were placed on a grower ration at about 35 days of age (About 20 kg live weight). Upon reaching about 60 kg of live weight, the animals were switched to the finishing ration for the duration of the feeding trial. The fatty acid profiles of the animal fat and test oils are reported in Table 1. The pigs were slaughtered at about 100 kg live weight (90 days on trial) at a local facility and chilled overnight (2°C). Carcasses were transported to the University of Georgia Meat Lab and the hams were removed (48 hr postmortem) and deboned and separated into top ham (Semimembranosus and Adductor), bottom ham (Biceps femoris and Semitendinosus) and other soft tissue (subcutaneous fat, seam fat and minor muscles). The top and bottom hams were defatted, vacuum-packaged in Cryovac[R] BH620 bags (Cryovac, Division of W.R. Grace and Co., Duncan, SC) and frozen at −34°C for a maximum of 45 days prior to processing. Hams were thawed at 2°C for 24 hr and green weights of the top ham and the bottom ham were determined using a Mettler[R] PM30 scale (Mettler Instrument Corp., Highstown, NJ). Hams were pumped to approximately 115% of their green weight with an Injectomat Automatic Bone-in Pickle Injector (E. Suter Machine Factory, Koelliken, Switzerland) using a 53° brine which contained 59.5g of sodium nitrite, 250.0g of sodium erythorbate, 59.5g of sodium tripolyphosphate, 3401.9g of sugar, 6803.9g of salt and 37797.8g of water. Pumped weights were determined and the hams were tumbled 4 hr in a Röchermatic TU 120 vacuum massager (Robert Reiser & CO., Inc. Canton, MA) at −0.95 atm pressure. Following tumbling, top and bottom hams were weighed, recombined and placed in flat knit ham bags and allowed to equilibrate 18 hr at 2°C and reweighed. Hams were cooked to an internal temperature of 65.5°C in an Alkar Food Processing Oven (Alkar Corp., Division of DEC International Inc., Lodi, WI) using a three-phase processing schedule. The three phases of the processing schedule were: (1) 3.5 hr at wet bulb (wb) 4.4°C and dry bulb (db) 60.0°C, (2) 4.0 hr at 53.3°C wb and 68.3°C db, with smoke, and (3) 61.6°C wb nd 73.8° db until hams reached internal temperature 65.5°C. Hams were weighed upon removal from the oven and chilled at 2°C for 24 hr and reweighed. Hams were vacuum-packaged and stored at −34°C a maximum of 45 days before analysis.

Fatty acid profile determination

Fatty acid profiles were determined on duplicate samples of the *Longissimus dorsi* to determine the effect of dietary fatty acid modification on intramuscular fat. The samples (2g) were homogenized in chloroform-methanol (2:1) and neutral lipids were extracted according to Folch et al. (1957). Extracted lipids were converted to methyl esters according to Shapierco (1975) and analyzed for individual fatty acids using a Perkin-Elmer (Model #8500) (Perkin-Elmer, Norcross, GA) gas chromatograph packed with Sp-2330 on a Supelco Column (Supelco Inc., Supelco Park, Bellefonte, PA). Inlet temperature was 230°C, column temperature was 200°C and detector temperature was 230°C. Flow rate of carrier gas (nitrogen) was 30 cm^3 /min.

Ham yield determination

Ham yields at various processing levels were determined as follows: top pump yield = (pumped top ham wt/green top ham wt) x 100%; bottom pump yield = (pumped bottom ham wt/green bottom ham wt) X 100%; overall pump yield = (pumped ham wt/green ham wt) x 100%; top massage yield = (massaged top ham wt/pumped top ham wt) x 100%; bottom massage yield = (massaged bottom ham wt/pumped bottom ham wt) x 100%; overall massage yield = (massaged ham wt/pumped ham wt) x 100%; smokehouse yield = (hot cooked ham wt/massaged ham wt) x 100%; cooled smokehouse yield = (cooled cooked ham wt/massaged ham wt) x 100%; total yield = (cooled cooked ham wt/green ham wt) x 100%.

Table 1—Fatty acid composition (%) of animal fat, safflower oil, sunflower oil and canola oil

Treatment	14:0	16:0	16:1	18:0	18:1	18:2	18:3
Animal fat	1.17	17.8	3.4	10.2	45.3	18.2	2.0
Safflower oil	0.09	5.4	0.0	2.2	72.1	19.4	0.0
Sunflower oil	0.06	4.1	0.0	4.0	80.9	9.5	0.0
Canola oil	0.09	4.7	0.4	1.7	57.7	22.5	12.4

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Table 2—Effect of dietary treatment on fatty acid composition (%) of the Longissimus dorsi

Treatment	14:0	16:0	16:1	18:0	18:1	18:2	18:3
Control	1.7[a]	23.8[a]	4.6[a]	11.7[a]	47.4[c]	6.7[d]	1.5[b]
Animal fat	1.7[a]	22.5[b]	4.2[b]	11.1[a]	44.6[e]	11.5[a]	1.6[b]
Safflower oil	1.7[a]	21.7[bc]	3.6[c]	10.0[b]	48.8[b]	10.4[b]	1.4[b]
Sunflower oil	1.7[a]	21.0[bc]	3.9[bc]	9.4[b]	51.7[a]	8.4[c]	1.5[b]
Canola oil	0.7[a]	20.6[c]	3.6[c]	9.8[b]	45.9[d]	12.3[a]	3.0[a]
SEM[f]	0.1	0.3	0.1	0.3	0.4	0.3	0.1

[a-e] Means in same column with like superscripts are not different (P > 0.05).
[f] Standard error of the mean.

Trained flavor sensory panel

An eight-member sensory panel was selected and trained according to procedures outlined by Cross et al. (1978). Four training sessions were held in which panelists were served portions of the Semimembranosus and the Biceps femoris from a variety of treatments to familiarize panelists with a wide range of scores. Training was completed when all panelists felt comfortable with the scoring system and were in close agreement. Panelists scored the ham on a 1 to 8 point scale for juiciness, first impression tenderness, overall tenderness, saltiness, flavor, flavor intensity and overall palatability (8 = extremely juicy, tender, tender, salty, flavorful, intense and palatable and 1 = extremely dry, tough, tough, nonsalty, unflavorful, bland and unpalatable). In addition, panel members indicated whether the ham was too salty, not salty enough or had off-flavor. Panelists were served 1 cm^3 portions of room-temperature ham. Panelists were alternated between eating samples of the Semimembranosus muscle and samples of the Biceps femoris muscle to prevent biasing of data.

Trained visual sensory panel

An eight-member trained visual panel scored the ham under 50 ft-c of light on a 1 to 8 point scale for slice breakage, lean color, uniformity of color, lean firmness and lean texture (8 = no breakage, extremely dark red, uniform, firm and fine and 1 = 100% breakage, extremely light pink, two-toned, soft and coarse). Four training sessions were held in which panelists evaluated ham from a wide variety of treatments to familiarize them with a wide range of scores. Training was completed when all panelists felt comfortable with the scoring system and were in close agreement. Members of the visual panel did not serve on the flavor panel.

Objective texture determination

Warner Bratzler shear force (kg/g) was determined by shearing ham cores (2.5 cm diameter) removed from 1.3 cm thick slices. One core from the Semimembranosus and one core from the Biceps femoris from the ham of each animal was sheared parallel to muscle fiber alignment.

Ham cores (as described above) were equilibrated at 25°C prior to being compressed twice to 70% of their original height with an Instron Model 1122 Universal Testing Machine (Instron Corp., Canton, MA) equipped with a Microcon computer. Full scale load was set at 50 kg, chart paper at 50 mm/min and the crosshead at 50 mm/min. Hardness, springiness, cohesiveness and chewiness were determined according to Bourne (1978).

Sodium and nitrite determination

Sodium content was determined with a Sodium Selective Ion Electrode (Model 13-620-501) and a Double-Junction Reference Electrode (Model 13-620-273) attached to a Fisher Accumet[R] 950 pH/ISE Meter (Model 13-636-950, Fisher Scientific Inc., Springfield, NJ). Nitrite content was determined according to AOAC (1984) procedures. All determinations were in duplicate.

Statistical analysis

Yield, visual panel, sodium and nitrite data analyzed as a function of five dietary treatments while sensory and objective texture data were analyzed as a function of five dietary treatments and two muscle sources and the interaction of dietary treatment with muscle source. An analysis of variance (Steel and Torrie, 1980) was used to analyze all dependent variables according to procedures outlined by SAS (1985). When the main effect or interaction was significant, means were separated by a comparison of least-squares means (Montgomery, 1984). The predetermined level of significance of $P < 0.05$ was used for all comparisons and will be used for the remainder of this report.

RESULTS & DISCUSSION

Fatty acid profiles

The fatty acid profiles of Longissimus dorsi indicated that oleic acid increased in hams from the sunflower and safflower oil treatment but decreased from the canola oil and animal fat treatments (Table 2). The decrease in percentage oleic acid in muscle tissues from pigs fed canola oil was partially due to increased proportion of polyunsaturated fatty acids.

Processing yields

Dietary treatment did not affect any yield parameter investigated (Table 3). Because practically all subcutanious and intermuscular fat was removed from the hams prior to processing, dietary fatty acid composition did not affect yield. In a companion study conducted in our laboratory, thaw loss, cooking loss and total loss of pork loin chops were not affected by dietary fatty acid modification. Dietary treatment did not affect the ability of the hams to bind and retain brine.

Trained sensory panel

Juiciness, saltiness, flavor, flavor intensity, and overall palatability were not affected by dietary treatment (Table 4). This suggested that fatty acid composition of diets of growing-finishing swine might be altered without affecting sensory characteristics of boneless hams. Since all subcutaneous and intermuscular fat was removed from the hams, they were extremely low in fat content, and this probably explains why dietary fatty acid composition did not affect the majority of sensory parameters investigated. However, first impression tenderness and overall tenderness scores were lower for hams from the control and the canola oil treatment than from the

Table 3—Effect of dietary treatment on processing yields of hams[a]

Yield parameter[b]	Control	Animal fat	Safflower oil	Sunflower oil	Canola oil	SEM[c]
Top pump yield	116	114	118	117	118	2
Bottom pump yield	110	110	109	110	107	1
Pump yield	113	112	113	114	112	1
Top massage yield	100	100	100	100	100	1
Bottom massage yield	100	100	100	99	100	1
Massage yield	100	100	100	100	100	1
Smokehouse yield	81	81	82	82	83	1
Cooled smokehouse yield	81	79	81	80	81	1
Total yield	91	90	92	91	92	1

[a] No significant differences (P > 0.05).
[b] Top pump yield = (pumped top ham wt/green top ham wt) × 100%. Bottom pump yield = (pumped bottom ham wt/green bottom ham wt) × 100%. Overall pump yield = (pumped ham wt/green ham wt) × 100%. Top massage yield = (massaged top ham wt/pumped top ham wt) × 100%. Bottom massage yield = (massaged bottom ham wt/pumped bottom ham wt) × 100%. Overall massage yield = (massaged ham wt/pumped ham wt) × 100%. Smokehouse yield = (hot cooked ham wt/massaged ham wt) × 100%. Cooled smokehouse yield = (cooled cooked ham wt/measured ham wt) × 100%. Total yield = (cooled cooked ham wt/green ham wt) × 100%. All values are % yield.
[c] Standard error of the mean.

Table 4—Influence of dietary treatment and musce type on sensory panel scores of hams[d]

	Juiciness	First impression tenderness	Overall tenderness	Saltiness	Flavor	Flavor intensity	Overall palatability	Off-flavor[e]
Treatment								
Control	5.5[a]	6.2[b]	6.4[b]	4.8[a]	5.5[a]	5.2[a]	5.3[a]	2.5[c]
Animal fat	5.2[a]	6.7[a]	6.8[a]	4.7[a]	5.6[a]	5.3[a]	5.4[a]	6.6[bc]
Safflower oil	6.1[a]	6.6[a]	6.8[a]	5.5[a]	5.7[a]	5.8[a]	5.6[a]	7.9[bc]
Sunflower oil	5.5[a]	6.7[a]	6.8[a]	4.8[a]	5.2[a]	5.2[a]	5.2[a]	13.9[b]
Canola oil	5.8[a]	6.1[b]	6.3[b]	4.9[a]	5.0[a]	5.4[a]	5.0[a]	25.2[a]
SEM[f]	0.2	0.1	0.1	0.2	0.2	0.2	0.4	3.6
Muscle								
Biceps femoris	5.4[b]	6.3[b]	6.5[b]	4.8[a]	5.3[a]	5.3[a]	5.2[a]	13.8[a]
Semimembranosus	5.9[a]	6.6[a]	6.7[a]	5.1[a]	5.5[a]	5.5[a]	5.4[a]	8.6[a]
SEM[f]	0.1	0.1	0.1	0.1	0.1	0.1	2.2	

[a-c] Means in the same column bearing like superscripts are not different ($P > 0.05$).

[d] Scored on a 1 to 8 point scale (8 = extremely juicy, tender, tender, salty, flavorful intense or palatable and 1 = extremely dry, tough, tough, unsalty, unflavorful, bland or unpalatable).

[e] Off-flavor is the percentage of panelists detecting off-flavors.

[f] SEM is the standard error of the mean.

animal fat, safflower oil and sunflower oil treatments. A higher percentage of panelists detected off-flavors in the canola oil treatment samples than in those of all other treatments. Twenty-five percent of the panelists detected off-flavors in the canola oil treatment sample as compared to 2.5% for control samples. The off-flavor of the canola oil treatment hams can be linked to derivatives of linolenic acid. Capillary volatile profile analysis showed a higher concentration of 2-pentenal and 2,4-heptadienal in bacon from the canola oil treatment (Shackelford et al. 1990). Trained sensory panelists detected off-flavors more frequently in bacon produced from swine fed 10% canola oil than those produced from swine fed 10% animal fat, safflower oil and sunflower oil. Sixty-five percent of the panelists detected off-flavors in the canola oil bacon as compared to 25 % for the canola oil ham. This suggested reduction in fat content and increased brine pump level alleviated most of the off-flavor problem of the canola oil treatment.

The Semimembranosus muscle scored higher for juiciness, first impression tenderness and overall tenderness than the Biceps femoris. However, note that the difference between the two muscles for each of these was less than one-half of a point (8-point rating scale). Thus, the difference is probably not of practical importance and would not warrant use of blade tenderization to improve tenderness of bottom ham. In beef, the Semimembranousus muscle was reported tougher than the Biceps femoris (Ramsbottom and Strandine, 1948). Muscle source did not affect saltiness, flavor, flavor intensity and overall palatability. Dietary treatment and muscle source did not interact to affect any of the sensory parameters.

Objective texture determination

Warner-Bratzler shear force values were highest for the hams from safflower oil and canola oil treatments and lowest for those from the animal fat and sunflower oil treatments (Table 5). Hardness values were highest for the canola oil treatment samples and the control and lowest for hams from the animal fat and safflower oil treatments. Springiness, cohesiveness and chewiness were not affected by treatment.

Warner-Bratzler shear force, hardness and chewiness were greater for the Biceps femoris while the Semimembranosus was more springy. These data closely paralleled sensory panel data for tenderness. No difference was seen between muscles for cohesiveness. Dietary treatment and muscle source did not interact to affect any objective texture parameters.

Table 5—Influence of dietary treatment and muscle type on objective texture parameters of hams

	Warner-Bratzler shear force (kg/g) $\times 10^{-2}$	Hardness (kg)	Springiness (cm) $\times 10^{-2}$	Cohesiveness $\times 10^{-2}$	Chewiness
Treatment					
Control	48[ab]	31.4[a]	54[a]	40[a]	6.7[a]
Animal fat	42[b]	29.1[b]	54[a]	41[a]	6.4[a]
Safflower oil	53[a]	28.6[b]	53[a]	39[a]	5.9[a]
Sunflower oil	46[b]	31.1[ab]	51[a]	39[a]	6.3[a]
Canola oil	53[a]	33.6[a]	50[a]	41[a]	6.6[a]
SEM[c]	02	1.2	01	01	0.3
Muscle					
Biceps femoris	52[a]	32.3[a]	52[b]	40[a]	6.7[a]
Semimembranosus	45[b]	29.2[b]	53[b]	40[a]	6.0[b]
SEM[c]	01	0.8	01	01	0.2

[ab] Means in same column within treatments with like superscripts are not different ($P > 0.05$)

[c] Standard error of the mean.

Table 6—Effect of dietary treatment on the visual panel scores of hams[a]

Visual parameter[b]	Control	Animal fat	Safflower oil	Sunflower oil	Canola oil	SEM[c]
Slice breakage	6.3	6.8	6.8	6.7	6.1	0.5
Lean color	5.5	5.8	5.8	5.7	4.9	0.3
Uniformity of color	5.8	5.8	5.2	5.9	6.0	0.4
Lean firmness	7.7	7.7	7.5	7.3	7.3	0.2
Lean texture	7.3	7.5	7.1	7.4	7.4	0.3

[a] No significant differences ($P \geq 0.05$).

[b] Scored on a 1 to 8 point scale (8 = no breakage, extremely dark red, uniform, firm or fine and 1 = 100% breakage, extremely light pink, two-toned, soft or coarse).

[c] Standard error of the mean.

Table 7—Effect of dietary treatment on sodium and nitrite content of ham[a]

Treatment	Sodium (mg/100g)	Nitrite (ppm)
Control	665.1	10.7
Animal fat	619.2	13.6
Safflower oil	691.3	18.0
Sunflower oil	652.8	18.5
Canola oil	615.9	11.7
SEM[b]	33.6	2.3

[a] There were no significant differences ($P \geq 0.05$).

[b] Standard error of the mean.

Trained visual panel

No differences were reported across treatments for lean color, uniformity of color, lean firmness, lean texture and slice breakage (Table 6). This indicated the cure ingredients and thermal processing alleviated differences inquality of raw hams from different dietary treatments. However, in a companion study conducted in our laboratory, differences were found between the five dietary treatments for cured lean color, fat color, oiliness, firmness and slice breakage of bacon.

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Cholesterol Content of Restructured Pork/Soy Hull Mixture

N. IBRAHIM, N. UNKLESBAY, S. KAPILA, and R.K. PURI

ABSTRACT

The effect of convective heat processing was studied on cholesterol and its oxides in a restructured pork/soy hull model system. Products were analyzed from 30 different time-temperature conditions of processing. Following lipid extraction, GC-MS was used for analysis of cholesterol and its oxides. No cholesterol oxides were found at 2 ppm in the product with any of the 30 time-temperature profiles, ranging from 19 –89.6 min of heat processing, and 9–97°C final product temperatures. Cholesterol in heat-processed samples ranged from 45.43–66.47 mg/100g. Positive correlations were found between final temperatures and fat and cholesterol content.

INTRODUCTION

FOODS CONTAINING CHOLESTEROL are subject to oxidizing conditions during various stages of processing. Exposure to elevated temperatures in contact with air for even a brief period is likely to lead to the formation of cholesterol oxides (Maerker and Unruh, 1986). Over 60 oxides resulting from cholesterol autoxidation, photoxidation, and enzymatic action have been described (Smith, 1981). Several cholesterol oxides have been implicated in adverse human health effects including cytoxicity, atherogenicity, mutagenicity and carcinogenicity. Cholesterol oxides have been reported in meat products (Rayan et al., 1981; Fioriti and Sims, 1967; Park and Addis, 1986a, 1986b). Since sterol oxidation is a free-radical process, any sterol-containing food exposed to pro-oxidant should be suspected as a possible source of oxidized dietary sterols (Finocchiaro and Richardson, 1983).

Restructured pork/soy hull products are being designed to add dietary fiber and reduce the fat and food energy contents of pork. Three plant sterols, with potential cholesterol lowering affects have been reported in soy hulls (Ibrahim et al., 1990). No researchers have reported the cholesterol content and extent of cholesterol oxidation in these mixtures. A systematic study to determine food processing and environmental factors which might facilitate sterol oxidation was needed. Our research was designed to study the effect of 30 time-temperature conditions on the cholesterol and cholesterol-oxide content of a restructured pork/soy hull mixture.

MATERIALS & METHODS

LABORATORY PROCEDURES were developed to give 30 different time-temperature curves in the mixtures, simulating those which would occur in various portions of a pork/soy hull mixture during convective heat processing. These curves were obtained by precisely controlling the amount and rate of convective heat transfer to the product. The final product temperatures achieved by the internal portion of the mixtures were altered. Procedures were designed to keep variables constant: homogeneous pork/soy hull mixture; mass, size, and initial temperature of mixture; location of product in the oven; oven temperature; and position of thermocouples. Given that this experimental control would produce almost identical time-temperature curves per replication, the preparation of composite samples would be feasible.

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Heat processing

Pork shoulder (27.3 kg) from the same lot of hogs fed a regular diet was obtained. The meat tissue and visible fat were separated and ground (Hobart Model K 5-A) with screen size of 6.0 mm. Ground tissue and fat were mixed so the total mixture would contain ca. 17% fat. The fat content was determined using the Babcock Method. The mixture was portioned (520g), wrapped with freezer paper and stored at −12°C for not more than 3 wk. Each portion was tempered at 5°C for 24 hr before formulation. Crushed soy hulls were obtained from the Archer-Daniel Midlands Soybean Processing Plant (Mexico, MO) and used as received. The size of the crushed soy hulls was determined (Table 1) with a Rotap Shaking Sieve Tester (Taylor Model No. 11404). Although beyond the scope of this research, particle size has been shown to affect moisture and lipid absorption of soy hulls (Muzilla et al., 1989).

The pork/soy hull mixture was prepared in two steps. First, crushed soy hulls (10 g), mild BBQ rub seasoning and sauce mix (4.8g), (Milwaukee Seasoning Laboratories, Inc., Germantown, WI), and 2% milk (40g) were mixed and allowed to set 15 min to enable the soy hulls to absorb all liquid. The ground pork (100 g) and salt (1g) were mixed in a Hobart mixer (Model K5-A, Troy, OH) 5 min at speed #2. The hydrated soy hulls were added and the mixing continued 15 min. Flexible reusable cold packs (Premium Fresh Farms, Balimar, CA) were positioned around the bowl to control product temperature during mixing.

Procedures were developed to obtain 30 different time-temperature profiles of the pork/soy hull mixture. A galvanized steel sheet metal box (10 x 10 x 6 cm) was constructed to enable placement of a mesh screen, 1 cm from the bottom and five layers (10 x 10 cm) of the mixture, each 1 cm thick. Each layer was stacked alternately with nylon mesh (10 x 10 cm) to enable their separation after heat processing. Five thermocouples (Type K) attached to a Data Logger (Omega Model OM-500), were placed through holes in one side of the box, such that they were located in the center of each of the five layers. This container was then placed in a larger box (26 x 26 x 14 cm) filled from the bottom to a level of 8 cm with vermiculite (Grace Zonolite Construction Product, Cambridge, MA). Additional vermiculite was added to insulate all sides of the small box except the top. A cover was placed on top of the large box. A center opening (10 x 10 cm) enabled the surface of the top layer to remain uncovered.

The large box was placed in an electric non-preheated forced air convection oven (Lang Co-20). The surface of the meat layers was located in the geometric center of the oven cavity. The box was heat processed until the fourth layer from the bottom reached one of six temperatures: 20°, 32°, 44°, 56°, 68° or 77°C. The surface temperature was monitored with an infrared pyrometer (Omega model).

With this procedure, heat was transferred to the surface of the top layer by convective heat transfer and an insignificant amount of radiation heat transfer from the oven walls (Skjoldebrand, 1985). Once heat reached the surface of the mixture, it penetrated into the top layer and was transferred by conduction. Because the sides and bottom of the small box were sufficiently insulated, all of the layers were heated by conduction from the top, with the exception of the surface of the top layer. As product denaturation and shrinkage occurred, moisture and fat collected below the wire mesh at the bottom of the small box. The top surface was exposed to air and reached higher surface temperatures; both factors associated with the formation of cholesterol oxides (Maerker and Unruth, 1986).

This procedure simulated the heating profiles which would occur throughout a pork roll, roast or patties when placed on a rack and

Table 1—Sieve sizes for crushed soy hulls

U.S. sieve (Size)	Soy hulls remaining (%)
20	24.5
25	11.9
35	24.1
>35	39.5

heated in a convection oven. This procedure enabled control of the amount of heat transferred to the center of each of the five stacked layers. Other researchers (Prusa and Hughes, 1986; Morgan et al., 1988) have analyzed the cholesterol content of pork tissues after they have been heat processed to a given internal product temperature. Their procedures disregarded the fact that pork tissue samples had different time-temperature profiles from those where their thermocouple was located. An advantage to the procedures we used is that precise time-temperature of the tissue was known. After heat processing, each layer was separated, weighed, vacuum-packaged (Multivac AC 800, Wolfertschwenden, W.G.), and stored at −45°C in a covered aluminum foil pan.

Composite samples

Five replications were performed for each of the six temperatures. Linear regression analysis (SAS, 1985) was used to analyze the time-temperature curves by layer and final temperature. R-values were in excess of 0.95. Thus, it was feasible to combine replications into composite samples. Thirty composite samples were formed by combining product from the center of each layer, according to the position of the layer in the box and ending temperatures. Cores, five cm in diameter, were taken from the center of each layer. The cores were divided into four equal parts which were randomly assigned to moisture, fat or cholesterol analysis.

Analyses

Moisture content was determined by the vacuum oven method 7.003 (AOAC, 1984). Analysis of cholesterol and its oxides were performed on both heat processed and unprocessed formulae. A 5 g aliquot of the formula was taken and enhanced with 40 ng of 5 α-cholestane as interal standard. The enhanced sample was extracted with chloroform:methanol mixture (2:1) by Folch procedure (Folch et al., 1957). This method has been shown to extract higher percentages of total lipids than procedures which use ether as solvent (Rhee et al., 1988). The total lipids content of the sample was determined gravimetrically after removing the solvent under vacuum. Cholesterol and related components were then separated from saponifiable fat through a procedure described by Park and Addis (1987). Saponification was carried out with 2NKOH in methanol. The nonsaponifiable fraction was extracted with ether (3x30 mL). Each fraction was washed with 0.5 KOH/MeOH and 3 times with 10 mL distilled water. The ether extracts were combined and concentrated to 1 mL under vacuum and transferred to amber vials. The residual polar fraction of lipids was removed by passing the extract through basic aluminum column (24 x 1.5 cm). The column was eluted with 100 mL of methanol:chloroform (9:1). The cleaned extract was concentrated to dryness and silylated according to procedures described by Park and Addis (1986a). The derivitizing agent and pyridine solvent were removed under a gentle stream of nitrogen, residue dissolved in hexane (1 mL) and analyzed by capillary gas chromatograph interfaced to an F.I.D. and confirmation of the analyses were by GC/MS, (mass spectograph).

Gas chromatographic analyses were carried out with DB-1 capillary column 50 m x 0.25 mm 1.D (J & W Scientific, Inc., Rancho Cordova, CA). Helium was used as a carrier gas at 8 p.s.i. Oven temperature was programmed for 100–265° at 5°/min rise. Confirmatory analyses were carried out with GC/MS System on Model 5890/5970 H.P. and Model 5100/Finnigan Corporation.

Statistical methods

Five replications were performed for each of the six ending temperatures, giving 30 composite samples (5 layers x 6 temperatures) for analyses. General linear regression procedures were performed on the time-temperature curves. Pearson product moment correlations (SAS, 1985) were determined among the final product temperatures and the moisture, fat and cholesterol contents.

RESULTS & DISCUSSION

HEAT PROCESSING conditions for the 30 composite samples are given in Table 2. The mean heat processing times varied from 19 to 89.6 min while the final internal temperatures varied from 8.6 to 97°C. No trends were identified for cholesterol content for any heat processing conditions.

The chromatogram of a typical enhanced sample is shown in Fig. 1B. The order of elution was identified as: 5 α-cholestane, cholesterol, epoxide, 7β-hydrocholesterol, triol, 7-ketocholesterol, and 25-hydroxycholesterol, respectively. The response of cholesterol and cholesterol oxide was found to be linear over a concentration range of 10-150 ng/μL for oxides and 50-100 ng/μL for cholesterol.

Typical chromatograms (Fig. 1) confirmed no cholesterol oxides were identified in any heat processed sample. The detection limit of the GC/MS used was 2 ppm for the analyses. This was found for all composite samples, heat processing times and final internal temperatures (Table 2). In addition, the surface temperature of the top layer, in contact with the air in the oven reached 128°C. These conditions simulated

Table 2—Heat processing conditions and cholesterol content of restructured pork/soy mixture

Conditions			Food Components				
				Fat		Cholesterol mg/100g	
Mean time (min)	Mean final temp. (°C)	Layer[a]	Moisture (%)	(%)	(% dry basis)	Wet Basis	Dry Basis
19	8.6	2	64.6	13.9	39.3	40.2	113.7
	10.5	3	65.6	12.0	34.9	34.9	101.4
	10.6	1	63.7	11.2	30.8	36.8	101.4
	20.3	4	63.1	13.0	35.2	36.6	99.2
	49.5	5	62.1	12.1	31.9	44.7	118.0
31.4	15.3	2	63.4	13.1	35.8	37.0	101.2
	19.2	1	63.4	12.4	33.8	32.4	88.3
	20.2	3	64.1	14.7	40.9	52.1	145.1
	32.2	4	63.5	13.4	36.7	50.1	137.3
	65.7	5	61.0	16.0	41.0	43.3	111.2
43.6	24.0	2	63.4	13.2	36.1	42.9	117.4
	26.5	1	64.1	12.4	34.5	36.4	101.2
	28.6	3	63.5	12.2	33.4	51.1	105.0
	44.2	4	63.0	13.7	37.1	42.4	114.8
	73.4	5	58.8	14.8	35.9	59.6	144.8
56.2	35.5	2	64.9	13.4	38.2	52.6	149.9
	40.2	1	63.8	13.0	36.0	64.4	178.0
	41.3	3	63.7	13.9	38.3	58.1	115.8
	56.1	4	61.7	14.0	36.5	55.5	144.8
	86.3	5	56.6	14.0	32.2	66.5	153.1
71.2	48.2	2	63.8	12.7	35.1	41.3	114.1
	52.7	3	63.3	12.5	34.0	40.4	110.1
	53.8	1	63.7	11.9	32.7	40.7	111.9
	68.1	4	61.4	14.4	37.3	33.9	88.0
	94.3	5	56.7	15.2	35.1	45.4	103.9
89.6	61.0	2	61.7	17.6	46.0	50.8	132.6
	62.8	1	61.5	13.0	33.8	47.3	123.1
	64.8	3	60.7	20.0	50.8	56.0	142.3
	77.0	4	58.2	16.9	40.4	46.5	111.3
	97.0	5	52.9	21.0	39.7	53.0	112.6

[a] Refers to position of mixture layer in the box (1 = bottom; 5 = top).

Table 3—Quantitative data of cholesterol and cholesterol oxides in enhanced sample (50 ng/each oxide + 250 ng cholesterol)

Compound name	Determined value (ng/uL)	Total amount (ng/uL)	Recovery (%)
Cholesterol	663	718	92
Epoxide	49.2	50	98
7B-hydroxycholesterol	53.9	50	108
Triol	52.2	50	104
7-ketocholesterol	40.2	50	80
25-hydroxycholesterol	55.3	50	112

Table 4—Correlation coefficients among components and ending product temperature of restructured pork/soy hull mixtures[a]

Variable	Fat (%)	Cholesterol (mg/100g wet basis)	Cholesterol (mg/100g dry basis)	Final product temp (°C)
Moisture (%)	−0.687*	−0.383**	−0.004	−0.865*
Fat (%)		0.381**	0.143	0.622*
Cholesterol (mg/100g wet basis)			0.923*	0.454**
Cholesterol (mg/100g dry basis)				0.141

[a] Refers to values for the 30 composite samples.
* Significant at $P < 0.05$.
** Significant at $P < 0.01$.

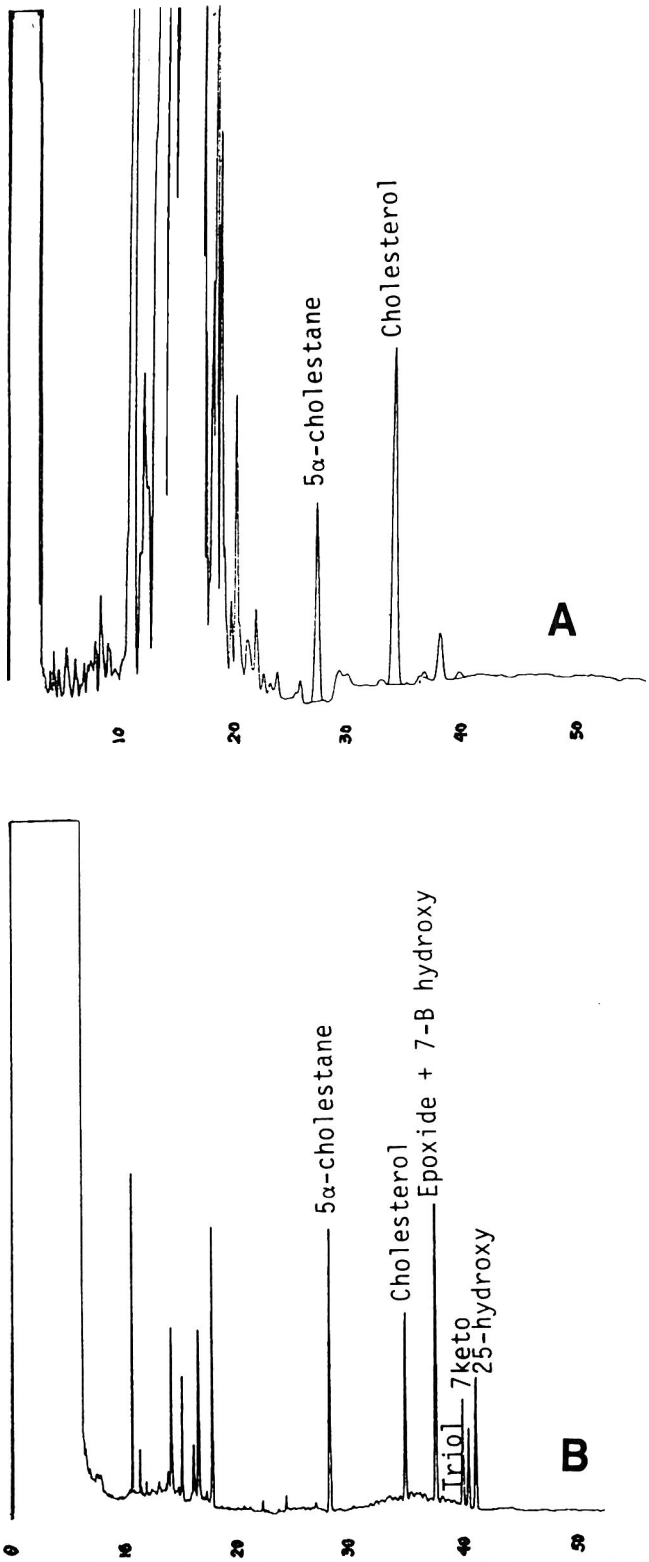

Fig. 1—(A) Chromatogram of plain sample of pork/soy hulls after heat processing. (B) Chromatogram of enhanced sample of pork/soy hulls after heat processing.

practically all conceivable time-temperature relationships that would be encountered during convective heat processing of a restructured pork/soy hull product. Cholesterol oxides did not form under these conditions of thermal processing and vacuum, frozen storage, at a level of 2 ppm or above. The amount of sterol recovered through the sample saponification and extraction is shown in Table 3. Since the percent recovery for most sterols was fairly high, no correction was applied to percentage recovery.

The value for cholesterol of the raw mixture was 46.3 mg/100g on a wet basis. This was lower than reported for ground pork: i.e. 59.1 mg/100 g (Punwar and Derse, 1978), 60.0 mg/100g (Feeley et al., 1972); 61.6 mg/100g (Tu et al., 1967) and 70.0 mg/100g (Ockerman, 1980). The lower cholesterol content in the formula can be attributed to the hydrated soy hulls which represented 32% of the total formula. Thus, cholesterol content could reasonably be lowered by ca. 25%.

Some correlation coefficients among the components and ending temperature for 30 composite samples are given in Table 4. A significant ($p<0.05$) correlation was found between the ending moisture and fat contents, as expressed on a percentage basis. This finding agreed with data reported by Reitmeier and Prusa (1987) who found percent fat content of ground pork increased as moisture content decreased.

Soy hull constituted 32% of the total formula in the sample and contributed in reduction of cholesterol content. Cholesterol oxides were not detected in any heat processed samples or storage samples above the detection limit of 2 ppm. Further research is in progress to investigate the antioxidant role of the soy hulls or other parameters during processing restructured pork/soy hull mixtures.

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Effect of Moisture Content on Density, Heat Capacity and Conductivity of Restructured Pork/Soy Hull Mixtures

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ABSTRACT

Sixteen restructured pork/soy hull mixtures were studied. Incorporation of unprocessed and processed and two particle sizes of hydrated soy hulls in the mixture resulted in moisture contents ranging from 74% to 85%. Standard laboratory procedures were used to measure density, heat capacity and thermal conductivity. Thermal diffusivity was calculated as a mathematical function of these three properties. Thermal diffusivity values were affected the most ($1.14–2.01 \times 10^{-7}$ m^2/sec) by moisture, followed by heat capacity (2.75–4.18 J/g°C), and thermal conductivity ($4.80–5.72 \times 10^{-3}$ W/cm°C). No significant changes in density occurred (1.02–1.06 g/cm^3).

INTRODUCTION

KNOWLEDGE of thermal properties such as conductivity, heat capacity and diffusivity are essential to design efficient food processes and processing equipment (Szczesniak, 1983; Singh, 1982; McProud and Lund, 1983). Okos (1987) stated that although academic and industrial personnel recognize the need for thermal property data, it is often difficult to find. A lack of information on such thermal properties has hindered development of food processing equipment (Hill et al., 1967) and process design for food products (McProud and Lund, 1983).

As Nesvadba (1982) pointed out, for newly developed food products, direct measurement is the optimal method for obtaining thermal property data. When the food product consists of several ingredients, direct measurement is even more important since values reported in the literature rarely cover complex foodstuffs. While the literature provides data on thermal properties of pork muscle (Meat Research Institute, 1972; Lentz, 1961), no thermal property data are available for soy hulls or restructured pork/soy hull products. Such products have been developed to produce pork products with reduced calories and cholesterol and increased fiber and minerals for human consumption (Ibrahim et al., 1990a). Further, plant sterols which may have further cholesterol-lowering effects for humans, have been identified in soy hulls (Ibrahim et al., 1990b).

Physical structure, density, and moisture are factors affecting thermal properties of foodstuffs (Mohsenin, 1980). When dealing with foodstuffs composed of particulate matter, particle size of the material also affects thermal properties (Jasansky and Bilanski, 1973). These factors, in addition to the complex chemical and physical composition of products, must be considered when measuring and interpreting thermal properties of restructured pork/soy hull mixtures.

Our investigation was undertaken to determine the effect upon selected thermal properties (density, heat capacity, thermal conductivity, and thermal diffusivity) when the moisture content of a restructured pork/soy hull product was altered. Moisture content was varied through incorporation of hydrated, processed (lignin-reduced) or unprocessed soy hulls using two particle sizes.

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MATERIALS & METHODS

Moisture content

The moisture content of dry soy hulls was determined by the air oven method (14.004, AOAC, 1984); that of ground pork, the vacuum oven method (7.003, AOAC, 1984). The mean percent moisture (± standard deviation) of the soy hulls varied for each particle size: 10.60 ± 0.19 (unprocessed fine grind); 11.40 ± 0.11 (unprocessed coarse grind); 8.05 ± 0.06 (processed fine grind); and 8.37 ± 0.06 (processed coarse grind). The mean percent moisture (± standard deviation) of pork was 73.72 ± 0.38. These values were used to calculate the percent moisture of each restructured pork/soy hull mixture (Table 1).

Preparation of product

Two types soy hulls (Quincy Soybean Co., Quincy, IL) were used. One had been processed to reduce lignin content (Quinsoy); the other was unprocessed. The soy hulls were ground through a FitzMill comminutor (Model DASO-6, Fitzpatrick Co., Elmhurst, IL) to produce finely ground (0.84 mm) or coarsely ground (2.36 mm) particles.

The hulls were hydrated (5 min) until saturated. The maximum water which was absorbed by the soy hulls at 25°C was determined previously (Muzilla et al., 1989a, b): 4.18g water/g unprocessed soy hull (fine grind); 5.15g water/g unprocessed soy hull (coarse grind); 7.00g water/g processed soy hull (fine grind); and 8.68g water/g processed soy hull (coarse grind).

Ground (3.0 mm) lean pork shoulder (ca. 6% fat, Folch et al., 1957) and salt (1% of pork) were added to the hydrated soy hulls and mixed 30 sec. The formulas (Table 1) were prepared daily at random, covered and held at ambient temperature (23–25°C) for no longer than 4 hr until thermal tests were conducted.

Density

A known mass (ca. 5g) of sample (24.8 ± 2.4°C) was added to a 60 mL volumetric flask which was filled to volume with distilled water (22°C). Density was calculated using the following equation:

$$\rho = \frac{M}{60 - V} \quad (1)$$

Table 1—Moisture end soy hull content of restructured pork/soy hull mixtures

Soy hull					
Type	Particle size	Mixture no.	Calculated moisture (%)	Dry soy hull (%)	Rehydrated soy hulls:pork (g/g)
Unprocessed	Fine (0.84 mm)	1	73.72	0.00	–
		2	74.25	2.50	0.15
		3	75.52	5.00	0.35
		4	76.78	7.50	0.64
		5	78.04	10.00	1.09
	Coarse (2.36 mm)	1	73.72	0.00	–
		6	74.74	2.25	0.16
		7	76.49	4.50	0.39
		8	77.60	6.75	0.65
		9	79.99	9.00	1.26
Processed	Fine (0.84 mm)	1	73.72	0.00	–
		10	75.47	2.00	0.19
		11	77.96	4.00	0.48
		12	80.44	6.00	0.93
		13	82.92	8.00	1.80
	Coarse (2.36 mm)	1	73.72	0.00	–
		14	75.96	1.75	0.21
		15	78.93	3.50	0.52
		16	81.91	5.25	1.04
		17	84.88	7.00	2.12

Table 2—Mean values for thermal properties of restructured pork/soy hull mixtures (25°C)[a]

Mixture no.	Thermal Property: Density (g/mL)	Heat capacity (J/g°C)	Thermal conductivity (× 10^{-3}W/cm°C)	Thermal diffusivity (× 10^{-7} m²/sec)
1	1.05 ± 0.01[b]	4.05 ± 0.15[d]	5.08 ± 0.15[b]	1.19
2	1.05 ± 0.01[b]	3.58 ± 0.15[c]	4.86 ± 0.15[b]	1.29
3	1.04 ± 0.01[b]	3.05 ± 0.15[b]	4.88 ± 0.15[b]	1.54
4	1.06 ± 0.01[b]	3.03 ± 0.15[b]	5.29 ± 0.15[b]	1.65
5	1.05 ± 0.01[b]	3.08 ± 0.15[b]	5.21 ± 0.15[b]	1.61
1	1.05 ± 0.01[cd]	4.05 ± 0.15[c]	5.08 ± 0.09[b,c,d]	1.19
6	1.03 ± 0.01[b]	3.58 ± 0.15[b]	4.80 ± 0.09[b]	1.30
7	1.04 ± 0.01[bc]	3.44 ± 0.15[b,d]	5.05 ± 0.09[b,c]	1.41
8	1.06 ± 0.01[d]	3.07 ± 0.15[d]	5.14 ± 0.09[c,d]	1.58
9	1.03 ± 0.01[b]	3.71 ± 0.15[b,c]	5.34 ± 0.09[d]	1.40
1	1.05 ± 0.01[b]	4.05 ± 0.13[b,c]	5.08 ± 0.11[c,d]	1.19
10	1.05 ± 0.01[b]	4.08 ± 0.13[b,c]	4.90 ± 0.11[c]	1.14
11	1.05 ± 0.01[b]	3.76 ± 0.13[b]	5.46 ± 0.11[b]	1.38
12	1.04 ± 0.01[b]	4.18 ± 0.13[c]	5.36 ± 0.11[b,d]	1.23
13	1.04 ± 0.01[b]	4.10 ± 0.13[a,c]	5.54 ± 0.11[b]	1.30
1	1.05 ± 0.01[b]	4.05 ± 0.16[b]	5.08 ± 0.09[b,c]	1.19
14	1.02 ± 0.01[d]	3.21 ± 0.16[c]	4.94 ± 0.09[b]	1.51
15	1.04 ± 0.01[bc]	3.08 ± 0.16[c]	5.30 ± 0.09[c]	1.65
16	1.03 ± 0.01[dc]	2.75 ± 0.16[c]	5.70 ± 0.09[d]	2.01
17	1.04 ± 0.01[bc]	3.00 ± 0.16[c]	5.72 ± 0.09[d]	1.83

[a] N = 5. Least square means ± standard error of means.
[b-d] Where superscripts ([b-d]) differ vertically in a column within a group, means are significantly ($P \leq 0.05$) different.

where ρ = density (g/mL); M = mass of sample (g); and V = volume of water added (mL) (Szczesniak, 1983). This procedure is recommended for meats. Precautions were taken for accurate measurements of the mixtures. Given that the soy hulls were saturated with water before they were added to the meat tissue, and the procedure was completed within 30 seconds, any moisture absorption by the sample was minimized.

Heat capacity

A 1 L Dewar flask was fitted with a plastic foam lid. A thermocouple (Type K) was placed through the lid to the midpoint of the thermos which had been calibrated with known amounts of hot and cold distilled water. A known mass (ca. 100g) of heated distilled water (ca. 80°C) was placed into the thermos and equilibrated. This temperature was recorded (Omega digital thermometer, Model 2176A). A known mass of sample (ca. 50g) at a known temperature (ca. 22°C) was added to the water and its temperature recorded after equilibration. The formula for calculating heat capacity was outlined by McProud and Lund (1983):

$$Cp = \frac{(CpH_2O)\,(M_w)\,(T_i - T_e)}{(M_s)\,(T_e - T_s)} \qquad (2)$$

where Cp = heat capacity of sample (cal/g°C); CpH_2O = heat capacity of water (cal/g°C) obtained from the calibration; M_w = mass of water (g); T_i = initial temperature of water in thermos (°C); T_e = equilibrium temperature of water and sample (°C); M_s = mass of sample (g); and T_s = temperature of sample (°C). For these procedures, the mean temperature of the samples was 24.8 ± 2.4°C. Values were converted to J/g°C by multiplying by the factor 4.1868 (Toledo, 1980).

Thermal conductivity

A small thermal conductivity probe (Sweat et al., 1973) containing a resistance heater was powered by a D.C. power supply set at 4.0 volts with current in the heater circuit ranging from 235-240 mA. An ice water filled thermos functioned as the reference junction for the thermocouple (Type T) located inside the probe. After each sample (ca. 43 g; 24.8 ± 2.4°C) was placed into a 30 mL Pyrex beaker, the probe was inserted into the center until its length was covered. The power supply was activated and the temperature rise recorded. Elapsed time and temperature values were sampled at 6 sec intervals for ca. 4 min and the slope between each pair of points calculated. By examining the values of slope, the nonlinear segments of the curve were rejected. Linear regression was performed on the remaining values to determine the best fit which was used to compute thermal conductivity (Sweat and Haugh, 1974).

Thermal diffusivity

Thermal diffusivity was calculated mathematically once density, heat capacity, and thermal conductivity of the sample (24.8 ± 2.4°C) were known using the equation:

$$\alpha = \frac{K}{\rho\, Cp} \qquad (3)$$

where α = thermal diffusivity (cm²/sec); K = thermal conductivity (W/cm°C); ρ = density (g/mL); and Cp = heat capacity (J/g°C) (Singh, 1982). Values were converted to m²/sec by multiplying by 10^{-4}.

Statistical analysis

Five replications of each thermal property measurement were made for each of the 16 formulas and ground pork. Data from the raw pork were analyzed with each group of mixtures (Table 1), resulting in four sets of data each containing five formulas. Each data set was analyzed separately using general linear model procedures (SAS, 1985) with analysis of variance with least square means (Snedecor and Cochran, 1980). The linear statistical model included the effect of moisture. Mean differences were ascertained using Fisher's least significant difference (Chew, 1976). Linear regression was used to determine the relationship between moisture and density, heat capacity, and thermal conductivity.

RESULTS & DISCUSSION

Density

Values for the density of the mixtures ranged from 1.02 to 1.06 g/mL (Table 2), with a mean value of 1.04 g/mL. With respect to the mean, the individual values of density ranged ± 1.9%. There were no statistical ($P > 0.05$) differences among density values of the mixtures formulated with processed or unprocessed finely ground soy hulls. When coarsely ground soy hulls were used, statistical ($P \leq 0.05$) differences were noted among density values of the model systems with both processed and unprocessed soy hulls.

As moisture increased, there was a slight downward trend, probably due to lower density of soy hulls and water compared to pork. This trend was very minor and would not have a major impact on product formulation, processing design or equipment usage.

Heat capacity

The heat capacity of the mixtures ranged from 2.75 to 4.18 J/g°C, with all groups displaying some statistical ($P \leq 0.05$) differences among their respective heat capacity values (Table 2). The mean value was 3.42 J/g°C, with a range from −20% to +22%. These values initially decreased as hydrated soy hulls were added to the pork, but as moisture content increased, this decrease leveled off, and in some cases reversed. This was due to water since its heat capacity was greater than any other components in the model systems.

Linear regression analysis showed that for the mixtures made with processed finely ground soy hulls, moisture had no significant ($P > 0.05$) effect on heat capacity. For the other three groups of mixtures, there was a significant ($P \leq 0.05$) quadratic response to moisture.

One influence on heat capacity could be the chemical reactions taking place. Some pigments in pork changed from red (oxymyoglobin) to brown (metmyoglobin) very rapidly when soy hulls were added. This was more pronounced for the unprocessed soy hulls than for the processed ones. When a chemical reaction occurs, the heat absorbed during the reaction is not available to raise the temperature of the material. Further investigations of these chemical changes are needed.

Heat capacity values differ between the mixtures containing finely ground and coarsely ground processed soy hulls (Table 2). Grinding changed the surface area of the hulls: 50.30 cm^2/g (processed fine grind) vs 18.16 cm^2/g (processed coarse grind) (Muzilla et al., 1989b). It may also have changed the physical structure of the hulls which could account for the change in their ability to absorb heat without a concomitant change in temperature. Cadden (1987) has shown the microstructure of some plant fibers can change when ground.

Thermal conductivity

The range of values for thermal conductivity of the mixtures was somewhat narrow (Table 2). The mean value was 5.22×10^{-3} w/cm°C with a range of −8% to 10% around the mean. All groups of mixtures displayed statistical ($P \leq 0.05$) differences between thermal conductivity values except those formulas with finely ground unprocessed soy hulls. After an initial drop in thermal conductivity, probably due to incorporation of the hulls, thermal conductivity values rose as moisture increased. Water accounts for this trend since the thermal conductivity of water is higher than that of the other components. Several authors reported the dominating effect water can have on thermal conductivity values (Mohsenin, 1980; Sweat and Haugh, 1974). Thermal conductivity of apples (Lozano et al., 1979), commercial sausages (Ziegler et al., 1987) and meat emulsions (Timbers et al., 1982) increased as moisture content increased.

Linear regression analysis showed that for those mixtures with either processed or unprocessed coarsely ground soy hulls, moisture had a significant ($P \leq 0.10$) cubic response to thermal conductivity.

The microstructure of the model system mixture may account for some of the differences in thermal conductivity (Mohsenin, 1980; Yano et al., 1981). If the particles were in close proximity, as they would be with finely ground soy hulls, heat would have to travel through the soy hulls, preventing an increase in thermal conductivity. When particles of a mixture are less close, as they would likely be with coarsely ground soy hulls, heat could more easily advance around the ingredients, effectively raising the thermal conductivity of the mixture. Those mixtures with coarsely ground soy hulls tended to have higher thermal conductivity values than their counterparts with finely ground hulls.

Thermal diffusivity

The range for thermal diffusivity of the mixtures was somewhat narrow (Table 2). A higher thermal diffusivity value means heat travels more easily through the food material (Singh, 1982). When mixtures had unprocessed soy hulls, those with finely ground soy hulls tended to have somewhat higher thermal diffusivity values than those with coarsely ground hulls (Table 2). Conversely, for mixtures made with processed soy hulls, those with the finely ground soy hulls tended to have lower thermal diffusivity values than those mixtures with coarsely ground hulls. These trends are important because differences in thermal diffusivity could play a major role in food formulation and optimal process and equipment design since such differences affect thermal processing times and temperatures.

Because thermal diffusivity is a mathematical function of the other three thermal properties, their composite trends are reflected in the trends for thermal diffusivity (Table 2). Thermal diffusivity values tended to increase when: (1) moisture increased, (2) thermal conductivity increased, and (3) heat capacity and density decreased. Other factors such as differences in chemical composition and spatial arrangement of the soy hulls in the model systems could account for some of the variation (Singh, 1982).

In summary, moisture, varied through incorporation of various types and sizes of soy hulls, affected thermal properties of the mixtures. Thermal diffusivity values were affected most (76% difference between the highest and lowest values), followed by heat capacity (52% difference) and thermal conductivity (19% difference). Study of thermal properties of additional mixtures with moisture contents within these ranges can provide regression curves which could lead to practical equations for predicting effects of moisture.

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Heat-induced Gelation of Myofibrillar Proteins and Sausages: Effect of Blood Plasma and Globin

K. AUTIO and F. MIETSCH

ABSTRACT

The gelation of meat myofibrils and sausages was monitored during heating with and without added blood globin and plasma by the dynamic rheological method. During heating of beef myofibrils (pH 6.5; prot conc 6.0%) the storage modulus (G') reached a minimum at 47.7 °C, decreased on further heating, and increased above 53 °C. At higher pH (6.9) the G' value was lower and the magnitude of tanδ increased substantially between 37 and 42 °C. Addition of blood globin and plasma changed the thermal gelation of myofibrils. During heating of control and protein-supplemented sausages, we observed two minor peaks in storage modulus in the range 40–55 °C and a steep increase at 55–80 °C. The storage modulus of low fat sausages, where 25% of meat was replaced by globin or plasma, was greater than that of control sausages.

INTRODUCTION

MUSCLE FIBRE is responsible for the fundamental structure of meat. Each individual fibre is composed of a number of myofibrils. When meat is comminuted, the myofibrils become shorter, the collagen and fat are broken down and a distribution of various sized particles becomes suspended in the aqueous phase (Egelandsdal and Mitchell, 1987). Myofibril possesses a similar protein composition to natural actomyosin, with the addition of a small amount of stroma protein. The gelling properties of actomyosin and myosin have been examined (Egelandsdal et al., 1986; Samejima et al., 1981) and reviewed by Ziegler and Acton (1984) and Asghar et al. (1985). However, the structure of myofibril is different from actomyosin. In myofibril, myosin molecules are assembled to form a thick filament and actin, tropomyosin and troponin form a thin filament. Few reports have been published on the gel formation of myofibril suspensions (Egelandsdal and Mitchell, 1987; Sano, 1988), which are important structural elements in comminuted meat products.

Soya and blood proteins are becoming increasingly popular as meat substitutes due to their lower cost than meat. Through differing widely in functional properties both are attractive additives for meat products (Hermansson and Tornberg, 1976; Auvinen and Puolanne, 1988). Globin powder has a high swelling capacity with low solubility (Autio et al., 1984), while plasma is highly soluble with minor swelling capacity (Hermansson and Tornberg, 1976).

Mechanical spectroscopy, which allows rheological characterization of a material without breaking the structure, can be used to monitor the structure of both model systems and more complex foods (Dea et al., 1983; Egelandsdal et al., 1986; Autio et al., 1989). Our objectives in this study were to: (1) monitor the gel-formation of beef and pork myofibrils, (2) monitor the structure formation of sausage a more complex meat system, and (3) study how globin and plasma proteins affect thermal gelation of meat myofibrils and sausages.

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Table 1 – Formulation for the sausages

	14.3% Fat				8.3% Fat		
	Control (g)	Globin (g)	Plasma (g)		Control (g)	Globin	Plasma (g)
Beef (5% fat)	60.0	39.0	39.0	Beef (5% fat)	92.2	46.1	46.1
Pork (50% fat)	60.0	60.0	60.0	Pork (3% fat)	92.2	92.2	92.2
Water	100.0	115.7	115.7		24.3	60.4	60.4
Fat					11.2	11.2	11.2
Salt	4.4	3.9	3.9		4.4	3.6	3.6
Added prot.		5.2	5.2			10.8	10.8
pH	5.8	6.2	6.2		5.5	5.9	5.8

MATERIALS & METHODS

Preparation of myofibrillar suspensions and sausages

Myofibrillar homogenates were prepared from beef and pork loin (m. longissimus dorsi) according to Harbitz et al. (1982). Batches from three different animals were used. Protein was determined by Kjeldahl nitrogen (AOAC, 1970). The method of Puolanne and Ruusunen (1978) was used to prepare sausages containing pork, beef, and either blood plasma or globin powder. The formulae for the sausages and the pH after protein addition are shown in Table 1. Plasma and globin powder (prepared by the method of Autio et al., 1985) were obtained from Provivo Oy (Finland). The chemicals were purchased from Merck AG (FGR) and were of analytical grade.

Rheological measurements

The rheological measurements were done with a Bholin Rheometer (VOR) in the oscillatory mode using a high temperature cell. Thermally conditioned gas was forced into the cell where the parallel plate geometry was located. The gap between the plates was set at 1 mm.

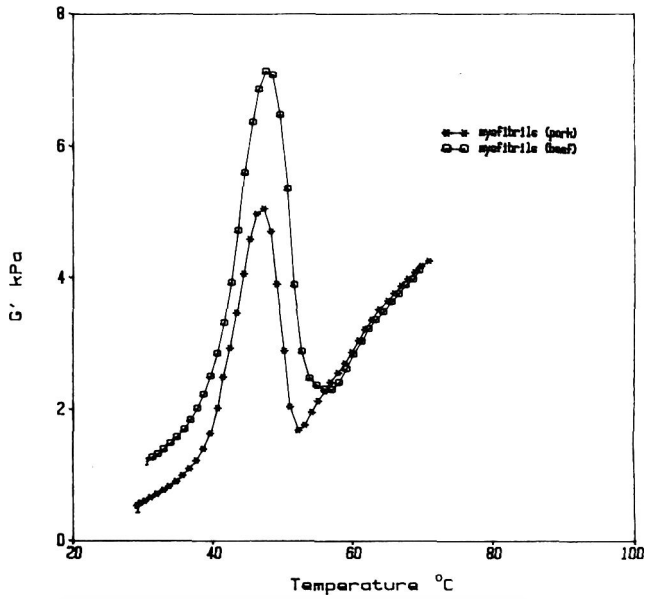

Fig. 1. – Rheologicla thermograms of a 5.4% pork (pH 6.6, 1.8% NaCl, batch II) and a 6.0% beef myofibril suspension (pH 6.5, 1.8% NaCl, batch III).

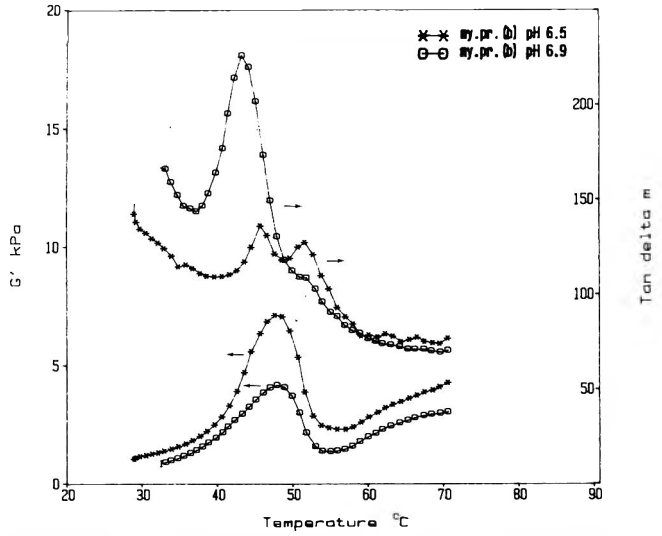

Fig. 2.—Effect of pH on the thermograms of a 6.0% beef myofibril suspension, with pH 6.5 and 6.9 (NaCl conc. is 1.8% in both suspensions, batch III).

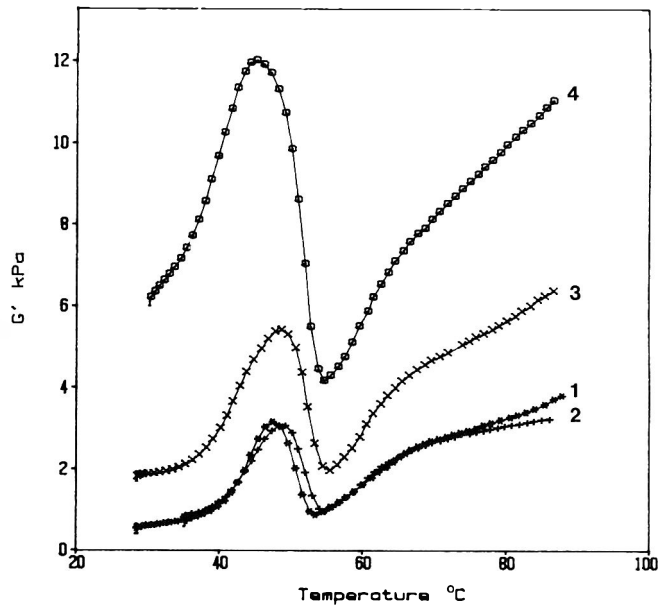

Fig. 3.—Effect of globin on the gelation of pork myofibrils (batch I). (1) 5.4% myofibril (MF) suspension (pH 6.4), (2) 5.4% MF + 2% globin (pH 6.9), (3) 5.4% MF + 5% globin (pH 7.3), (4) 5.4% MF + 10% globin (pH 7.5).

Drying of the sample was prevented by using an o-ring and silicon oil. The temperatures of the sample and the gas were measured by thermocouples.

Measurements were made by applying a small amplitude oscillation to the material (0.006 strain unit). The linearity was tested at the peak minimum. The amplitude ratio of the stress-to-strain gave the complex modulus, G*. The two sine waves had a phase difference, δ, which was used to give the storage and loss components, in-phase component being the storage or elastic component (G') and the quadrature component being the viscous or loss term (G''). The tanδ (= G''/G') was calculated from the storage and loss moduli. The frequency was 1 Hz and the heating rate 1 °C/min. Ross-Murphy (1988) gives an excellent overview of the small deformation technique, which we used, applied both to model and real food systems.

RESULTS & DISCUSSION

THE GELATION of suspensions of 5.4% porcine and 6.0% bovine myofibrillar proteins in 1.8% NaCl solution, at pH 6.6 and 6.5, respectively, is shown in Fig. 1. The storage modulus (G') increased on heating to 48 °C. On further heating it decreased, reaching a minimum at 52 and at 57 °C for pork and beef myofibrils, respectively, and then increased steadily up to 70 °C. Increasing pH decreased the G' (Fig. 2). It is well known that myosin forms a much stiffer gel at pH 6.2 than at pH 7.0 (Fretheim et al., 1985). At pH 6.9 the increase of G' between 37 and 42 °C was accompanied by a substantial increase in tanδ suggesting we were not dealing with gel-formation. G' increased from 42 to 48 °C and tanδ decreased, and from 48 to 52 °C both G' and tanδ decreased. In studies on the heat-induced gelation of 1-2.2% myofibril suspensions Egelandsdal and Mitchell (1987) attributed the drop in G' to soluble protein and myofibril protein aggregation. At pH 7.0 they found the gelling of soluble proteins to be poor, whereas at pH 6.2 it was sufficient to prevent precipitation of myofi-

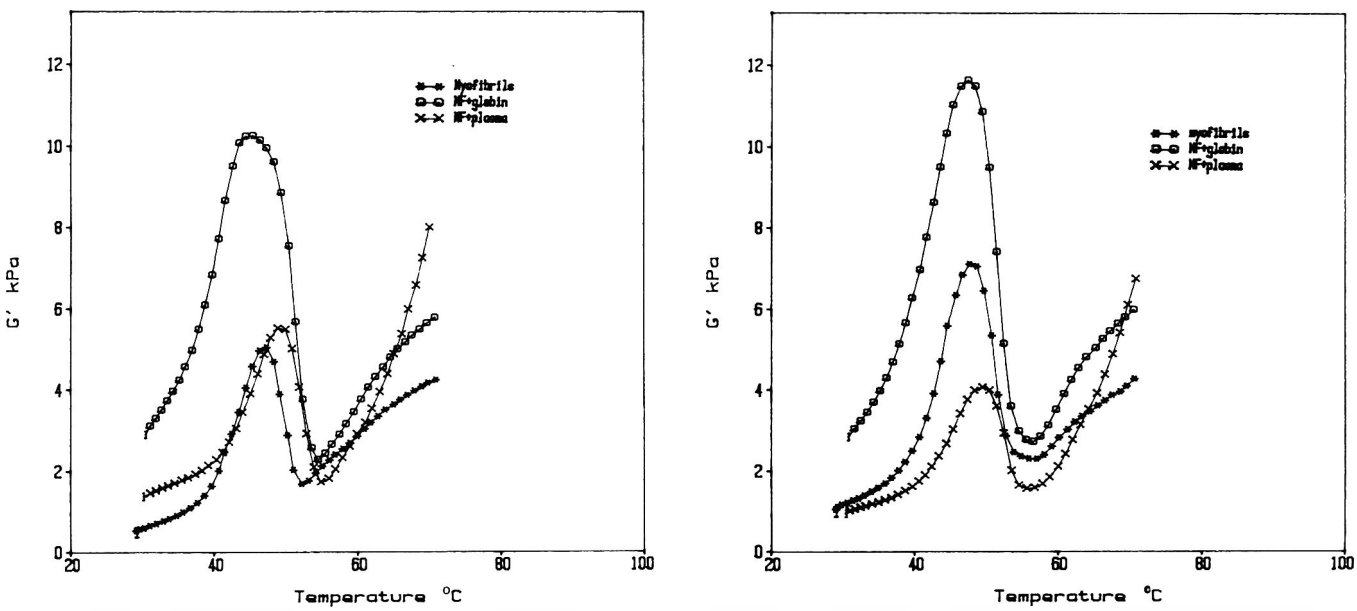

Fig. 4.—Effect of 5% globin and 5% plasma on the gelation of pork (a) and beef (b) myofibrils. (a) 5.4% pork myofibril (final pH 6.4, batch II) suspension supplemented with 5% globin (final pH 7.3) and 5% plasma (final pH 7.1). (b) 6.0% beef myofibril suspension (final pH 6.5, batch III); supplemented with 5% globin (final pH 7.3) and 5% plasma (final pH 7.1).

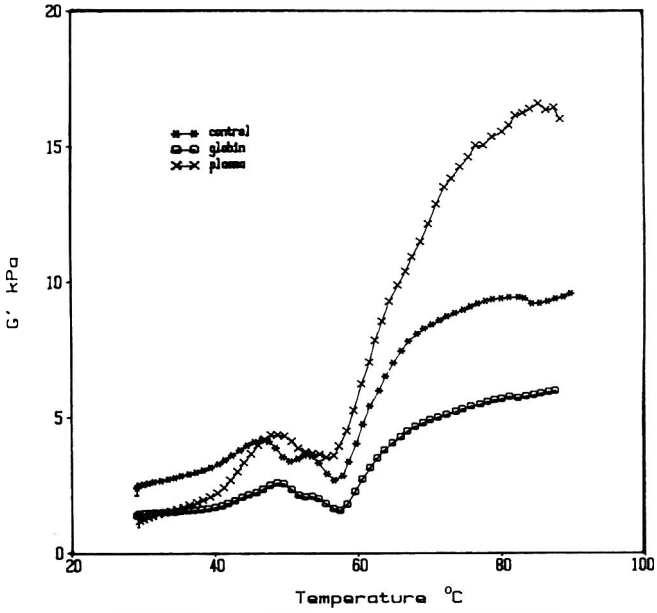

Fig. 5. – Thermograms of sausages with high fat content.

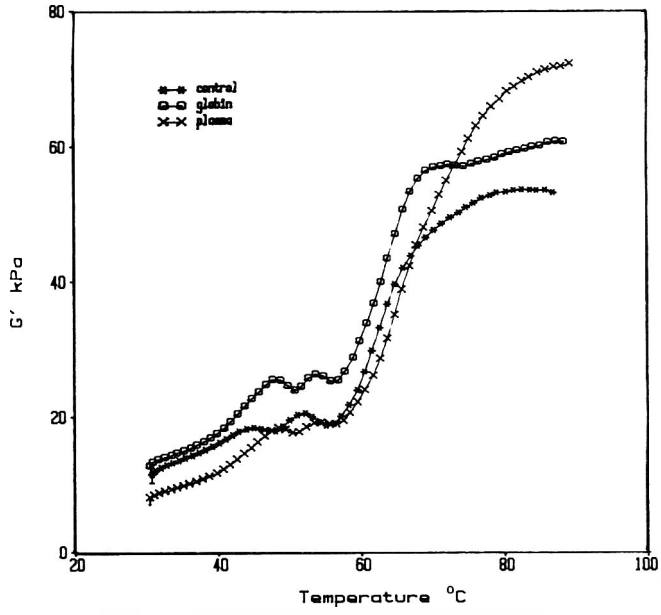

Fig. 6. – Thermograms of sausages with low fat content.

brils. The protein concentration of myofibril suspension was substantially higher in our study. At pH 6.9, between 48 and 52 °C, the elasticity of the system was increased at the same time as a marked loss of gel rigidity occurred. This structural change may be related to a transition from a filamentous to an aggregated structure. Explanation of these changes requires further investigation. Globin and plasma proteins altered the thermal gelation of myofibrils (Fig. 3, Fig. 4a and b). The globin powder increased the magnitude of G', though the heat-induced changes in G' were similar to those of myofibrillar suspension without globin. The addition of globin increased the final pH of the suspensions, as indicated in the legend of Fig. 3. The marked increase in G' caused by the addition of globin could not be a pH effect, since high pH decreased the G' of myofibrils (Fig. 2). Plasma powder behaved differently, with pork and beef myofibrils. Plasma proteins depressed the peak (T = 48°C) of beef myofibrils and increased that of pork relative to the myofibrils without added proteins. In both pork and beef the storage modulus increased after 60 °C, when plasma proteins started to form a gel.

The main textural changes in sausages during heating took place above 60°C (Fig. 5 and 6). These changes are related to the structural changes in myofibrillar proteins and collagen (Hermansson, 1986). The storage modulus was much lower in sausages with higher fat content (14.7%) supplemented with globin than in control sausages. Plasma proteins substantially increased the G' above 60 °C, just as with the myofibrils. Sausages with lower fat (8.3%) were much stiffer than the sausages with high fat. The two peaks in G' in the temperature range 45 to 55 °C were much greater in sausages supplemented with globin than in the other sausages. The first peak was more pronounced in sausages having high pork content and the second peak in sausages having high beef content (not shown). Probably these peaks originate from the structural changes of pork and beef myofibrils in sausages. At 80 °C the storage modulus of the sausages, where 25% of meat was replaced by globin and plasma, was higher than that of controls. Above 60 °C plasma increased the storage modulus of sausages with low fat content.

CONCLUSIONS

THE CHANGES in storage modulus of myofibrillar suspension with heating could be divided into three characteristic temperature stages: in the 30–48 °C range, the storage modulus increased dramatically; in the 48–57 °C range it decreased; and in the 57–80 °C range it again increased. Globin caused increase in the magnitude of G' at all temperatures, whereas plasma caused increases only above 60 °C. During heating of sausages two minor peaks in G' were observed at 40–55°C and a very steep increase 55–80°C. Plasma proteins increased the storage modulus above 60 °C regardless of fat content. Globin increased the storage modulus at all temperatures in sausages with low fat, but decreased it in sausages with high fat.

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Effects of Feeding Elevated Levels of Monounsaturated Fats to Growing-finishing Swine on Acceptability of Low-fat Sausage

S. D. SHACKELFORD, M. F. MILLER, K. D. HAYDON, and J. O. REAGAN

ABSTRACT

Sixty barrows and gilts were given one of five diets consisting of a control (corn and soybean meal), and four similar diets with a 10% replacement of animal fat, safflower oil, sunflower oil or canola oil. Pork trimmings, from carcasses of animals fed the five diets were used to formulate sausages at fat levels of 25 and 35% and water levels [4(% protein) + 3% and 4(% protein) + 11%]. Sensory panelists reported control sausages were springier than those from animal fat diets which were springier than those from all high oleate treatments. Sausages from high oleate treatments were more tender and less cohesive than the others. Sausage from canola oil treatment scored lowest flavor and palatability.

INTRODUCTION

SATURATED FAT is considered a primary cause of hypercholesterolemia (Mattson and Grundy, 1985). High blood cholesterol levels have been associated with atherosclerosis (Mattson, 1975, 1980). Thus, the reduction of saturated fat consumption has developed as a chief diet and health issue in regard to meat consumption. Diets high in monounsaturated fat have been associated with decreases in incidence of coronary heart disease. Keys (1970) reported the prevalence of heart disease was relatively low in certain areas of the Mediterranean region in which diets high in monounsaturates are typically consumed. Diets high in high oleate safflower oil have been reported to lower plasma cholesterol in man (Mattson and Grundy, 1985; Grundy, 1986).

Research is needed on the feasibility of producing sausage from the tissues of swine fed diets containing elevated levels of high oleate safflower oil, sunflower oil, and canola oil. Pork containing elevated levels of unsaturated fat has been shown to be different in processing characteristics (St. John et al., 1986). The effects of decreasing fat content and elevating water content of high oleate sausage should be investigated. The objectives of our study were to investigate the effects of feeding high oleate diets on the physical, chemical, and sensory attributes of low-fat, fresh pork sausage.

MATERIALS & METHODS

SIXTY BARROWS and gilts were given one of five dietary treatments consisting of a control diet of corn and soybean meal and four similar test diets that contained a 10% replacement of either animal fat, safflower oil, sunflower oil or canola oil. The fatty acid composition of the animal fat and test oils is presented in Table 1. The treatments were initiated when animals were placed on a grower ration at about 35 days of age (about 20 kg live weight). Upon reaching about 60 kg of live weight, the animals were switched to the finisher ration for the duration of the feeding trial. A detailed description of the diets and the feeding trial were reported by Miller et al. (1990). The pigs were slaughtered at about 100 kg live weight (90 days on trial) at a local facility and chilled overnight at 2°C. Carcasses were transported to the University of Georgia Meat Lab and fabricated 48 hr postmortem. Lean trim and fat trim were removed from the carcasses, vacuum-packaged in Cryovac® BH620 bags (Cryovac, Division of W.R. Grace and Co., Duncan, SC) and frozen at −34°C for 45 days. Lean and fat trim was ground through a 13 mm plate using a Butcher Boy Model A42.50 meat grinder (Lasar Mfg. Company, Inc., Los Angeles, CA). Trimmings were ground frozen, to decrease spoilage and refrozen after a sample was taken for determination of proximate composition.

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Product formulation and processing

Pork trimmings, from carcasses of animals given each of the five dietary treatments, were used to formulate sausage at two fat levels (25 and 35%) and two water levels [4(% protein) + 3% and 4(% protein) + 11%]. The proper amounts of lean trim, fat trim and ice were weighed and mixed with Pork Sausage Seasoning (Blend 64-T A. C. Legg. Co., Inc. Birmingham, AL). The sausage was mixed with a Butcher Boy Model 150F mixer (Lasar Manufacturing Company, Inc., Los Angeles, CA) ground through a 6.5 mm plate and stuffed into plastic casings (TP# 48980, TeePak Co., Inc., Chicago, IL). Sausage chubs were frozen at −34°C and sliced into 1.3 cm thick patties. Patties were vacuum-packaged and frozen at −34°C a maximum of 30 days for analysis.

Proximate composition

Values for moisture, fat (chloroform-methanol extractable component) and protein (Kjeldahl nitrogen) were determined using standard AOAC (1980) procedures. All determinations were in duplicate.

Fatty acid profiles

Fatty acid profiles were determined on duplicate samples of each sausage. The samples (2g) were homogenized in chloroform-methanol and neutral lipids were extracted according to Folch et al. (1957). Extracted lipids were converted to methyl esters according to Shapierco (1975) and analyzed for individual fatty acids using a Perkin-Elmer (Model #8500) (Perkin-Elmer, Norcross, GA) gas chromatograph packed with Sp-2330 on a Supelco column (Supelco Inc., Supelco Park, Bellefonte, PA). Inlet temperature was 230°C, the column temperature was 200°C and the detector temperature was 230°C. Flow rate of carrier gas (nitrogen) was 30 mL/min.

Trained sensory panel

An eight-member sensory panel was selected and trained according to procedures outlined by Cross et al. (1978). Four training sessions were held in which panelists were served sausage from a wide variety of treatments to familiarize panelists with a wide range of scores. Training sessions were concluded when panelists were in close agreement (i.e., individual raw scores did not vary more than ±1 from mean raw score and all panelists agreed they were comfortable with

Table 1—Fatty acid composition (%) of animal fat, safflower oil, sunflower oil and canola oil

Treatment	14:0	16:0	16:1	18:0	18:1	18:2	18:3
Animal fat	1.17	17.8	3.4	10.2	45.3	18.2	2.0
Safflower oil	0.09	5.4	0.0	2.2	72.1	19.4	0.0
Sunflower oil	0.06	4.1	0.0	4.0	80.9	9.5	0.0
Canola oil	0.09	4.7	0.4	1.7	57.7	22.5	12.4

Table 2—Proximate composition of fresh sausage formulated with high oleate pork

Treatment	Fat level (%)	Water level (%)	Protein (%)	Expected moisture (%)	Actual moisture (%)	Fat (%)
Control	25	3	14.0^{a}	59.0^{cd}	56.6^{ef}	25.6^{def}
	25	11	13.1^{c}	63.2^{a}	59.4^{c}	26.6^{d}
	35	3	12.4^{de}	52.4^{gh}	51.0^{i}	33.7^{ab}
	35	11	11.0^{h}	55.0^{f}	53.3^{g}	34.6^{a}
Animal fat	25	3	13.2^{c}	55.8^{f}	57.9^{de}	25.3^{def}
	25	11	12.5^{d}	61.0^{b}	60.9^{ab}	23.6^{f}
	35	3	12.5^{d}	53.0^{g}	54.3^{g}	30.2^{c}
	35	11	11.4^{gh}	56.4^{ef}	55.8^{f}	30.9^{c}
Safflower oil	25	3	14.0^{a}	58.8^{cd}	59.5^{bc}	24.1^{ef}
	25	11	12.1^{def}	59.4^{bcd}	61.5^{a}	25.2^{def}
	35	3	11.9^{efg}	50.6^{hi}	53.0^{gh}	32.1^{bc}
	35	11	9.9^{i}	50.6^{hi}	53.1^{gh}	34.6^{a}
Sunflower oil	25	3	13.8^{ab}	58.2^{de}	60.1^{bc}	23.3^{f}
	25	11	12.2^{def}	59.6^{bcd}	60.1^{bc}	25.3^{def}
	35	3	11.6^{g}	49.2^{i}	51.5^{i}	33.9^{ab}
	35	11	11.7^{fg}	57.8^{de}	56.4^{ef}	31.0^{c}
Canola oil	25	3	13.5^{bc}	56.8^{ef}	57.7^{de}	25.7^{de}
	25	11	12.3^{de}	60.2^{bc}	59.0^{cd}	27.1^{d}
	35	3	11.7^{fg}	49.8^{i}	51.4	33.9^{ab}
	35	11	9.9^{i}	50.6^{hi}	51.7^{hi}	35.4^{a}
SEM[j]			0.2	0.6	0.5	0.7

[a-i] Means in same column with like superscripts are not different ($P > 0.05$).
[j] Standard error of the mean.

the scoring system). Panelists sat in booths in an isolated room free of distractions. Green fluorescent lighting was used. Panelists were instructed to eat crackers and drink room temperature water and apple juice to cleanse the palate between samples. Panelists scored sausages on a 1 to 8 point scale for juiciness, springiness, tenderness, cohesiveness, texture, flavor and overall palatability (8 = extremely juicy, springy, tender, cohesive, coarse, flavorful or palatable and 1 = extremely dry, nonspringy, tough, noncohesive, fine, unflavorful or unpalatable). In addition, panel members indicated whether or not they detected any off-flavors or if sample were too tough, too mushy or too dry.

The sausage was cooked 3 min, flipped and cooked 6 min and flipped again and cooked 3 min using Farberware[R] Model B300 electric skillets (Farberware, Subsidiary of Kidde, Inc., Bronx, NY) calibrated to 148.9°C using a Molytek[R] Model 3702 potentiometer (Molytek Inc., Pittsburgh, PA) and a copper/iron thermocouple probe submersed in vegetable oil. The sausages were cut into 1 cm^3 portions and served to panelists while warm (about 65°C). Sausage was weighed before and after cooking to determine cooking yield using a Mettler[R] PC4400 balance (Mettler Instrument Corp., Highstown, NJ).

Yield determination

Cook yield was determined as follows:

$$\text{Cook yield} = (\text{Cooked pattie wt/raw pattie wt}) \times 100\%$$

Objective texture determination

Allo-Kramer shear force (kg/g) was determined by shearing sausage strips (2.5 cm wide) which were removed from the center of cooked patties. Six strips per treatment combination were equilibrated to 25°C and sheared using a multi-bladed Allo-Kramer shearing device attached to an Instron Model 1122 Universal Testing Machine equipped with a microcon computer (Instron Corp., Canton, MA). The full scale load was set at 100 kg, crosshead speed at 200 mm/min and chart speed at 50 mm/min.

Six cores (2.5 cm diameter) per treatment combination were removed from the center of cooked patties and allowed to equilibrate to 25°C before being compressed twice to 70% of their original height. The full scale load was set at 20 kg, crosshead speed at 50 mm/min and chart speed at 50 mm/min. Hardness, springiness, cohesiveness and chewiness were determined according to Bourne (1978).

Statistical analysis

Data were analyzed as a function of five dietary treatments, two fat levels and two water levels and all possible interactions. Analysis was conducted using the General Linear Models Procedure of the Statistical Analysis System (SAS, 1985). When interactions were not significant, they were removed from the model. When the main effect or interaction was significant, means were separated by comparison of least-squares means (Montgomery, 1984). The predetermined level of significance of $P < 0.05$ was used for all comparisons and will be used for the remainder of this report.

RESULTS & DISCUSSION

Proximate composition

Proximate composition of the sausages is presented in Table 2. Within each dietary treatment-fat level combination, except for the sunflower oil treatment at 35% fat, protein content was decreased by high-water treatments. Within the sunflower oil-35% fat formulation, added water level did not affect protein content. The actual moisture content of each formulation, except the control-25% fat-11% water formulation, was within 3% of expected moisture content, which was calculated by multiplying actual protein content of the formulation times four and adding the amount of water to the sum. The control-25% fat-11% water treatment could not reach the expected moisture level because the sum of moisture, protein and fat would have exceeded 100%. Within each dietary treatment-fat level combination, moisture content either increased or remained the same with the high-water treatment. The maximum difference in moisture content, between water levels, within a given treatment was 5%. Fat content was the same within all dietary treatment-fat level combinations except the safflower oil and sunflower oil 35% fat formulations. All of the 25% fat formulations had less fat than any of the 35% fat formulations. The 25% fat formulations averaged 25.2% while the 35% fat formulations averaged 33.0%.

Table 3—Effect of dietary treatment, fat and water on % fatty acid profiles of sausage formulated with high oleate pork

Fatty acid (%)	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1
Treatment								
Control	1.48^{a}	24.7^{a}	3.4^{a}	13.4^{a}	44.7^{a}	8.8^{a}	0.0^{c}	1.1^{c}
Animal fat	1.26^{b}	20.1^{b}	3.1^{b}	10.6^{b}	45.8^{d}	14.9^{b}	0.5^{b}	1.4^{a}
Safflower oil	1.08^{c}	17.3^{c}	2.2^{c}	7.7^{c}	55.1^{b}	13.5^{c}	0.0^{c}	1.2^{bc}
Sunflower oil	1.02^{c}	16.7^{d}	2.3^{c}	7.3^{d}	60.4^{a}	9.5^{d}	0.0^{c}	1.3^{b}
Canola oil	0.98^{c}	16.5^{d}	2.1^{d}	7.6^{cd}	50.4^{c}	15.7^{a}	4.5^{a}	0.0^{d}
SEM[f]	0.03	0.1	0.03	0.1	0.1	0.1	0.04	0.05
Fat level (%)								
15	1.17^{a}	19.1^{a}	2.6^{a}	9.4^{a}	51.1^{b}	12.5^{a}	1.0^{a}	1.0^{b}
25	1.16^{a}	19.0^{a}	2.6^{a}	9.3^{a}	51.4^{a}	12.5^{a}	1.0^{a}	1.1^{a}
SEM[f]	0.02	0.1	0.02	0.1	0.1	0.1	0.02	0.02
Water level (%)								
3	1.17^{a}	19.1^{a}	2.6^{a}	9.3^{a}	51.2^{a}	12.5^{a}	1.0^{a}	1.0^{a}
11	1.16^{a}	19.1^{a}	2.6^{a}	9.3^{a}	51.3^{a}	12.5^{a}	1.0^{a}	1.0^{a}
SEM[f]	0.02	0.1	0.02	0.1	0.1	0.1	0.02	0.02

[a-e] Means in same column within each main effect with like superscripts are not different ($P > 0.05$).
[f] Standard error of the mean.

Table 4—Influence of dietary treatment, fat and water on sensory panel scores of sausage formulated with high oleate pork[e]

	Juiciness	Springiness	Tenderness	Cohesiveness	Texture	Flavor	Overall palatability	Too mushy
Treatment								
Control	5.6[a]	5.1[a]	6.3[b]	5.1[a]	5 0[a]	6.8[a]	6.5[a]	6.3[b]
Animal fat	4.9[a]	4.2[b]	6.5[b]	4.6[a]	4 8[ab]	6.1[a]	5.4[ab]	2.1[b]
Safflower oil	5.5[a]	2.3[d]	7.6[a]	3.4[b]	4 3[c]	6.0[a]	4.2[bc]	35.3[a]
Sunflower oil	5.6[a]	3.2[c]	7.2[a]	3.6[b]	4 5[abc]	5.7[a]	4.7[bc]	23.1[a]
Canola oil	5.0[a]	2.5[cd]	7.5[a]	3.1[b]	4 4[bc]	4.4[b]	3.6[c]	24.8[a]
SEM[f]	0.3	0.3	0.2	0.3	0 2	0.5	0.4	5.2
Fat level %								
25	5.3[a]	3.6[a]	6.9[a]	4.2[a]	4 5[a]	6.1[a]	5.1[a]	16.9[a]
35	5.3[a]	3.3[a]	7.1[a]	3.8[a]	4 7[a]	5.5[a]	4.7[a]	19.7[a]
SEM[f]	0.2	0.2	0.1	0.2	0 1	0.3	0.3	3.3
Water level %								
3	5.0[b]	3.9[a]	6.7[b]	4.2[a]	4 7[a]	6.1[a]	5.2[a]	11.9[b]
11	5.7[a]	3.0[b]	7.3[a]	3.7[b]	4 5[a]	5.5[a]	4.6[a]	24.7[a]
SEM[f]	0.2	0.2	0.1	0.2	0 1	0.3	0.3	3.3

[a-d] Means in same column within each main effect with like superscripts are not different (P > 0.05).
[e] Scored on a 1 to 8 point scale (8 = extremely juicy, springy, tender, cohesive, coarse, flavorful, or palatable and 1 = extremely dry, nonspringy, tough, noncohesive, fine, unflavorful, or unpalatable). Too mushy is the percentage of panelists who felt the sample was too mushy.
[f] Standard error of the mean.

Table 5—Interaction of dietary treatment and water level on juiciness, shear force and hardness of sausage formulated with high pork[1]

Treatment	Water level	Juiciness	Shear force (kg/g)	Hardness (kg)
Control	3%	6.0[ab]	2.7[a]	10.7[a]
Control	11%	5.3[bcd]	2.4[b]	8.5[cd]
Animal fat	3%	4.3[d]	2.8[a]	9.9[ab]
Animal fat	11%	5.6[abc]	2.0[d]	7.5[de]
Safflower oil	3%	4.4[d]	2.1[c]	10.8[a]
Safflower oil	11%	6.5[a]	1.9[d]	7.8[de]
Sunflower oil	3%	5.5[abc]	2.4[b]	9.3[bc]
Sunflower oil	11%	5.6[abc]	1.8[de]	8.1[cd]
Canola oil	3%	4.7[cd]	1.5[f]	6.5[e]
Canola oil	11%	5.3[bcd]	1.7[e]	6.7[e]
SEM[g]		0.4	0.1	0.5

[a-e] Means in same column with like superscripts are not different (P > 0.05).
[f] Juiciness is on a 1 to 8 point scale (8 = extremely juicy and 1 = extremely dry).
[g] Standard error of the mean.

Table 6—Influence of dietary treatment, fat level and water level on cooking yield and objective texture data of sausage formulated with high oleate pork

	Yield (%)	Shear (kg/g)	Hardness (kg)	Springiness (cm)	Cohesiveness	Chewiness
Treatment						
Control	54.2[a]	2.5[a]	9.6[a]	0.66[b]	0.32[b]	2.1[a]
Animal fat	52.1[a]	2.4[a]	8.7[a]	0.64[b]	0.37[a]	2.1[a]
Safflower oil	54.1[a]	2.1[b]	9.3[a]	0.65[b]	0.37[a]	2.3[a]
Sunflower oil	54.7[a]	2.1[b]	8.7[a]	0.70[a]	0.38[a]	2.3[a]
Canola oil	55.0[a]	1.6[c]	6.6[b]	0.64[b]	0.36[a]	1.6[b]
SEM[1d]	1.8	0.05	0.3	0.01	0.01	0.1
Fat level (%)						
25	58.3[a]	2.0[b]	8.7[a]	0.67[a]	0.36[a]	2.1[a]
35	49.8[b]	2.3[a]	8.4[a]	0.65[a]	0.36[a]	2.0[a]
SEM[d]	1.1	0.03	0.2	0.01	0.01	0.1
Water level (%)						
3	56.3[a]	2.3[a]	9.4[a]	0.67[a]	0.36[a]	2.3[a]
11	51.7[b]	2.0[b]	7.7[b]	0.65[b]	0.37[a]	1.9[b]
SEM[d]	1.1	0.03	0.2	0.01	0.01	0.1

[a-c] Means in same column within each main effect with like superscripts are not different (P > 0.05)
[d] SEM = standard error of the mean.

Fatty acid profiles

Fatty acid profiles (Table 3) indicated the oleic acid (18:1) increased from that of the control in the animal fat, safflower oil, sunflower oil and canola oil treatments. Concomitantly, myristic acid (14:0), palmitic acid (16:0) and stearic acid (18:0) were highest for the control, intermediate for the animal fat treatment, and lowest for the safflower oil, sunflower oil and canola oil treatments. The ratio of monounsaturated fat to saturated fat (m/s) increased from that of the control in all experimental treatments. The polyunsaturated fat ranged from 9.6% in the control to higher levels in all the experimental treatments. The feeding of 10% high oleate sunflower oil to growing-finishing swine resulted in a 37% decrease in saturated fat and a 30% increase in monounsaturated fat of sausage and the level of polyunsaturated fat increased by only 7%. The ratio of oleic acid (18:1) to palmitic acid (16:0) increased from 1.8 in the control to higher values in all treatments. Fat level and water level changes did not result in any practical changes in fatty acid profiles.

Trained sensory panel

Sensory panel scores (Table 4) for juiciness were not affected by treatment while springiness scores were highest in the control, intermediate in animal fat treatment samples and lowest in the high oleate oil samples. Concomitantly, the oil treatments sausages were more tender and less cohesive than the controls or those from the animal fat treatment. The canola oil treatment samples received the lowest scores for flavor and overall palatability. These data coincide with work of St. John et al. (1986) which showed the level of canola oil in the diet (0, 10 or 20%) did not affect juiciness while feeding of 20% canola oil to swine resulted in frankfurters that were less springy and less palatable. Skelley et al. (1975) reported no difference in flavor of sausage produced from pigs fed roasted soybeans. The high oleate oil treatments sausages were rated "too mushy" more often than those of the controls or the animal fat treatments. Diet fat level did not affect any of the sensory parameters investigated. This is most likely due to the overriding effects of dietary treatment and water level. The cooking method possibly favored the low fat sausage as it may have been too harsh for the 35%-fat sausage. The high water sausage was juicier, less springy, more tender and less cohesive but was rated "too mushy" more often. Water level did not affect texture, flavor or overall palatability. Treatment and water level interacted to effect juiciness (Table 5). Within the control and the sunflower oil and canola oil treatments, juiciness was not affected by water level while within the animal fat and safflower oil treatments, juiciness increased as added water increased. These results indicated high levels of added water (11%) might be necessary to attain acceptable juiciness when producing sausage from swine fed animal fat or high oleate safflower oil because of the fat loss during cooking.

Yield determination

Cooking yield (Table 6) was not affected by dietary treatment. However, cooking yields were higher for the low fat treatment and lower for the high water treatment. Ahmed et

Table 7—Interaction of dietary treatment and fat level on objective texture parameters of sausage formulated with high oleate pork

Treatment	Fat level	Shear (kg/g)	Hardness (kg)	Springiness (cm)	Cohesiveness	Chewiness
Control	25%	2.2[c]	9.1[ab]	0.66[bc]	0.33[c]	2.0[bc]
Control	35%	2.9[a]	10.2[a]	0.65[c]	0.32[c]	2.2[abc]
Animal fat	25%	2.1[cd]	8.3[b]	0.59[d]	0.37[ab]	1.8[c]
Animal fat	35%	2.7[b]	9.0[ab]	0.70[ab]	0.37[ab]	2.3[ab]
Safflower oil	25%	2.0[d]	10.0[a]	0.66[bc]	0.37[ab]	2.5[a]
Safflower oil	35%	2.1[cd]	8.6[b]	0.64[c]	0.38[a]	2.1[bc]
Sunflower oil	25%	2.0[d]	8.3[b]	0.70[a]	0.37[ab]	2.1[abc]
Sunflower oil	35%	2.3[c]	9.1[ab]	0.70[ab]	0.40[a]	2.5[a]
Canola oil	25%	1.7[e]	7.9[b]	0.72[a]	0.39[a]	2.2[abc]
Canola oil	35%	1.5[e]	5.3[c]	0.57[d]	0.34[bc]	1.0[d]
SEM[f]		0.1	0.5	0.01	0.01	0.2

[a-e] Means in same column within treatment with like superscripts are not different ($P > 0.05$).
[f]SEM = standard error of the mean.

al. (1990) found similar results when investigating cooking yields of fresh pork sausage.

Objective Texture Determination

Allo-Kramer shear force (Table 6) was highest for sausages from the controls and the animal fat treatments, intermediate for those from the safflower oil and sunflower oil treatments and lowest from the canola oil treatments. Hardness and chewiness were lowest for the canola oil treatment samples. The safflower oil treatment sausage was springier than that of all other treatments while the control was least cohesive. The low fat sausage required less force to shear which indicated the method of cookery was too harsh for the 35% fat sausage and resulted in formation of a hard crust on the outside of the patties. Fat level did not affect hardness, springiness, cohesiveness or chewiness. The high water treatment resulted in decreased Allo-Kramer shear force, hardness, springiness and chewiness while water level did not affect cohesiveness. Treatment and fat level interacted to affect Allo-Kramer shear force, hardness, springiness, cohesiveness and chewiness (Table 7). Within the control, animal fat and sunflower oil treatments, Allo-Kramer shear force was lower for the low fat sausages while no difference was seen between fat levels within all other treatments. These results can best be explained by the fatty acid profiles of the sausages. The shear force values of the dietary treatments containing the highest levels of polyunsaturated fat (the canola oil and safflower oil treatments) were not affected by over-cooking to the degree of the sausages from the other dietary treatments. Within the control, animal fat and sunflower oil treatments, there was no difference in sausage hardness between fat levels, however, within the safflower oil and canola oil treatments, hardness was greater for the low fat sausage. Within the animal fat treatment, springiness was decreased by low fat while the converse was true in the canola oil treatment. Springiness was not affected by fat level within all other treatments. The low fat, canola oil sausage was more cohesive than the regular fat (35%) canola oil sausage while cohesiveness of all other treatments was not affected by fat level. The low fat, animal fat sausage was less chewy while within the safflower oil and canola oil treatments, the low fat sausage was more chewy. Chewiness of the control and the sunflower oil treatment was not affected by fat level.

Dietary treatment and water level interacted to affect shear force values and hardness (Table 5). Within the canola oil treatment, shear force values were higher for the high-water treatment while within all other treatments, Allo-Kramer shear force values were lower for the high water treatment. Hardness of the control, animal fat and sunflower oil treatment sausages was decreased by the high water treatment while hardness of the sunflower oil and canola oil treatment sausages was not affected by water level.

CONCLUSION

THE ADDITION of 10% high oleate sunflower oil to the diet of growing-finishing swine can help to decrease saturated fatty acids and increase monounsaturated fatty acids. High oleate oil dietary treatments may lower sensory panel scores for overall palatability of sausages produced at 35% fat. However, overall palatability scores of low fat sausages produced from swine fed high oleate safflower oil and sunflower oil were comparable to control sausages. Producing a highly palatable pork sausage with reduced total fat and increased monounsaturated/saturated fat ratios is feasible.

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Chemical and Sensory Studies of Antioxidant-Treated Beef

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ABSTRACT

Ground beef patties treated with metal chelators, free radical scavengers, rosemary and sodium alginate were examined by chemical (TBARS and gas chromatography) and sensory means in regard to warmed-over flavor (WOF). A highly trained analytical sensory panel evaluated the patties for desirable descriptors, such as cooked beef brothy (CBB) and for WOF descriptors, painty and cardboardy. Results showed many of the compounds retarded lipid oxidation when judged by chemical means, but not all affected development of WOF when judged by sensory means since CBB values decreased and WOF descriptors increased. The free radical scavengers appeared overall the most effective inhibitors of WOF.

INTRODUCTION

THIRTY YEARS AGO, Tims and Watts (1958) described warmed-over flavor (WOF) as the rapid development of an oxidized flavor in refrigerated cooked meat during storage. They further described the flavor characteristics as rancid or stale. Since then, many terms have been used to describe WOF. In a recent review, Melton et al. (1987) summarized several of these terms, e. g., sulfuryl, musty, stale, rancid, greasy, warmed-over, metallic, putrid, bitter, and sour. Johnsen and Civille (1986) developed a lexicon of terms to describe not only WOF but also desirable flavor notes. Love (1988) presented a modification of those descriptors while reporting factors used in selecting and training a panel for descriptive analysis of meat flavor. She further reported evaluation of descriptors for beef and for assessment of panelist performance.

Since the report of Tims and Watts (1958), the definition of WOF has expanded to include raw refrigerated stored meat and stored cooked meat not rewarmed. Over the past three decades, studies on WOF determined it was caused by lipid oxidation of phospholipids catalyzed by iron. These topics, and others related to WOF, have been covered extensively in comprehensive reviews (Pearson et al., 1977; Pearson and Gray, 1983; St. Angelo and Bailey, 1987; Asghar et al., 1988). Recent results from our laboratory (Vercellotti et al., 1987; Spanier et al., 1988; St. Angelo et al., 1988; Vercellotti et al., 1989a) have shown that WOF is not solely due to lipid oxidation. There is strong evidence that reactions involving protein degradation or heteroatomic compounds may also be implicated with WOF, particularly with the deterioration of desirable beefy flavor notes. Many of these reactions involve free radicals. Because of the complexity of the chemical reactions that occur during development of WOF, we question the validity of the term "warmed-over flavor", and suggest that a new more descriptive term is needed to replace the inadequate WOF term. However, the widely recognized and accepted term will be used in this report.

In spite of research conducted over the past three decades, the deterioration of freshly cooked flavor and increase in off-flavors particularly with fresh precooked foods are still problems confronting meat related industries. As described in the cited reviews, evidence suggests that lipid oxidation reactions are catalyzed by iron, metalloproteins and/or free radicals and these components are predominately involved in development of WOF. Consequently, various antioxidants, particularly chelators, have been used successfully, as judged by 2-thiobarbituric acid reactive substances (TBARS). In addition to chelator-type compounds, others that are free radical scavengers, or oxygen binders, have also been evaluated. However, in many of those studies, sensory studies were generally conducted without a highly trained analytical panel. Furthermore, many such compounds have their own flavor characteristics, and therefore, can adversely affect desirable flavor characteristics of the meat.

The objectives of our research were to investigate the WOF process in antioxidant-containing raw and cooked ground beef by chemical (TBARS) and instrumental (direct gas chromatography, GC) methods of analyses, to correlate those with other data from sensory methods and to determine by sensory evaluation if any additives used in prevention of WOF imparted other flavor characteristics.

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MATERIALS & METHODS

Food grade chemical additives

Carrageenans (44, Lactarin XP 4019 and Gelcarin SA 911) were supplied by Marine Colloids, Springfield, NJ. Rosemary, types W and O, were supplied by Kalsec, Inc., Kalamazoo, MI. Sodium alginate (Protanal SF 120) was supplied by Protan, Inc., North Hampton, NH. Gluconn-delta-lactone (GDL) was a gift of Finnsugar Biochemical, Inc., Schaumburg, IL. Tenox 20A, Tenox 4, tertiarybutylhydroquinone (TBHQ), and propyl gallate were supplied by or purchased from Eastman Chemicals, Kingsport, TN. DL-alpha-tocopheryl acetate was purchased from Grand Island Biological Co., Grand Island, NY. Sodium tripolyphosphate (STP), was a gift of FMC Corporation, Philadelphia, PA. Ascorbate-2-monophosphate, ascorbate-2-triphosphate, and ascorbate-2-mono-, di-, tri-, tetraphosphate were supplied by Dr. Paul Seib, Kansas State University, Manhattan, KA. Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), L-ascorbate, sodium salt, and ethylenediaminetetraacetate, tetrasodium salt, (EDTA) were purchased from Sigma Chemical Co., St. Louis, MO. Diethylenetriaminepentaacetic acid (DTPA) was purchased from Aldrich Chemical Co., Milwaukee, WI. All other additives were of the highest quality and were purchased from various suppliers.

Meat sample preparation

Top round roasts (Semimembranosus muscle), were purchased from a local supermarket the morning of sample preparation. After removing adipose fat, the lean meat was ground twice (1.0 cm plate followed by 0.8 cm plate) and separated into several 400g portions. Total fat ranged from 4–5%. From these portions, 80g (±0.5g) patties were made and placed into standard, 100 mm x 20 mm, glass petri plates with covers, and immediately frozen 3 days as standards. To other portions, antioxidants were dissolved or suspended in 5 mL deionized water and vigorously mixed 2 min with an Oster Blender. After thorough mixing, samples were made into 5 80g (±0.5g) patties and placed into the petri plates and allowed to stand (marinate) overnight, ca. 18 hrs, at 4°C. Water was added to control samples, and treated similarly to experimental samples. After marinating overnight, patties were cooked on a Farberware grill 7 min/side. Internal temperature was 65 ±1°C. Immediately after cooking, some patties were evaluated by trained sensory panel, followed by chemical and instrumental evaluations. Other samples were either frozen (−20°C) or stored in their respective petri plates in a refrigerator (4°C) 2 days to develop WOF.

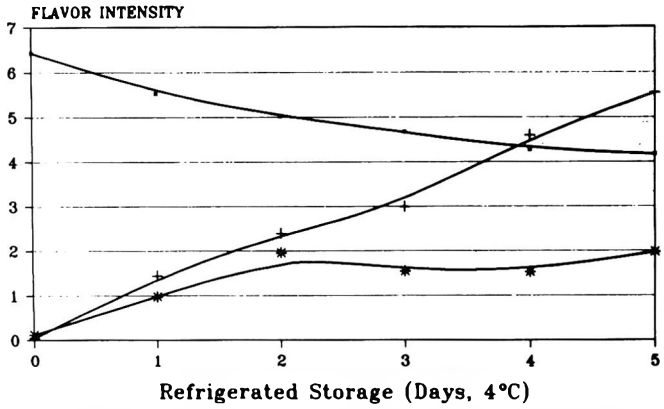

Fig. 1—Intensity of meat flavor characteristics, cooked beef brothy (●———●), painty (+———+), and cardboard (———*) in cooked patties stored at 4°C.*

On the day the samples were evaluated, the cooked refrigerated samples were rewarmed in an oven and evaluated by sensory or chemical methodologies. Standards, raw ground patties, were thawed, cooked, and evaluated as described above. In some instances, marinated samples were stored raw 2 days at 4°C or −20°C, cooked, and evaluated. For studies that involved 5-day storage, the patties which contained no additives, were stored in petri plates at 4°C.

Sensory analysis

An inhouse analytical sensory panel of 12-15 members was trained for descriptive analysis of WOF as described by Johnsen and Civille (1986) and more recently by Love (1988). The descriptive terms of Love (1988), were used. These were: cooked beef brothy (CBB), beef fat, browned, serum/raw, cooked liver, green grain/vegetable, cardboardy (CBD), painty (PTY), and burned/scorched. Tastes included sweet, sour, salty, and bitter. Mouthfeel included metallic and astringent. Sensory scores were presented as intensity values on a 15 point scale with 0 having the lowest intensity and 15 the highest (Meilgaard et al., 1987). After cooking or rewarming, each pattie was cut into 8 bite size wedges and served in standard (60 mm x 15 mm) glass petri dishes with covers. Each sample was assigned a 3-digit random number to mask identity. Panelists were seated in partitioned booths, and the room was lighted with red lights to mask color. The panelists received one sample at a time, and were instructed to rinse between samples which were cooked and served 5 min apart. The control samples and all concentrations of a specific additive were presented at one session to allow comparisons between concentrations. Two or more additives were evaluated at each session. The order of presentation was randomized to reduce position effect. All panel members were able to distinguish beef descriptors at a very high degree of proficiency and reproducibility. Standards (nothing added) were used for warm-up purposes and standardization of panelists.

Chemical analyses

The volatile profiles of meat samples were separated by direct GC using an external closed inlet device (ECID) on packed Tenax GC/PMPE columns as described by Dupuy et al. (1987) and by St. Angelo et al. (1987). Identification of volatile compounds was achieved with the system coupled to a Finnigan-MAT 4000 Gas Chromatograph/Mass Spectrometer/Data System as described. Basically, the method used the ECID for securing a small (0.84 x 8.5 cm) glass liner containing the ground meat samples (ca 1.2 grams) directly into the injection port of GC, heating the sample, and eluting the volatiles onto a packed column, as described by Legendre et al. (1979). From the GC profiles obtained, the degree of lipid oxidation was measured as the increase in integrator area counts for hexanal and for total volatiles. Duplicate samples were run and values were within 6% error. This method has been found highly reproducible at the 5-6% level (Dupuy et al., 1978; Vercellotti et al., 1989b).

The distillation method of Tarladgis et al. (1960) was used to analyze for 2-thiobartituric acid reactive substances (TBARS) on all patties. TBARS values were reported as mg malonaldehyde per kg sample, in duplicate, and the standard deviation calculated. A standard curve was prepared with 1,1,3,3-tetraethoxypropane (Aldridge Chem.). A Hewlett-Packard 8450A diode array spectrophotometer was used to measure TBARS at 532 nm.

RESULTS & DISCUSSION

Sensory descriptors of beef

Among the meat flavor descriptor notes, three were of special interest. The first was cooked beef brothy (CBB), which was the primary descriptor used to denote the highly desirable, freshly cooked, beefy flavor. The other two terms were used to describe WOF; these were painty (PTY) and cardboardy CBD). As WOF developed over storage 5 days at 4°C, the intensities of these descriptors were plotted as shown in Fig. 1. The flavor note, CBB, usually has a value of 6.5 upon cooking ground top round. As WOF developed, CBB decreased from 6.5 at day zero to about 4 at day 5. The other descriptors, PTY and CBD, were very low in freshly cooked (day zero) samples, but increased as WOF developed. After 2 days CBD leveled off, whereas PTY increased steadily.

WOF is a dynamic complex of ongoing flavor reactions. As reviewed by Liu et al. (1987), many researchers have suggested over the past two decades that the principal constituents of meat flavor and aroma were heteroatomic compounds containing oxygen, nitrogen and sulfur, present in parts per billion. Many such compounds recently have been isolated from cooked meat concentrates using a purge-and-trap system and identified by GC/MS (Vercellotti et al., 1987; Liu et al., 1987). In a separate study of sulfur containing heteroatomic compounds, the concentration of several of these decreased as boiled beef was stored 5 days at 4°C (St. Angelo et al., 1988).

PTY is known to correlate very well with hexanal. As PTY increased during WOF development, so did hexanal and TBARS values. The correlation coefficients between sensory scores and chemical or instrumental values and between TBARS and instrumental values showed very good agreement (St. Angelo et al., 1987). Dupuy et al. (1987) showed an appreciable increase in hexanal could be observed within a few hours after storage at 4°C. Thus, hexanal is an excellent marker for lipid oxidation, and WOF. Many other compounds increased in intensities with development of WOF (Dupuy et al., 1987; St. Angelo et al., 1987; Bailey, 1986); hexanal, however, is the most prominent. Recently, Spanier et al. (1988) showed by linear regression analysis a highly significant correlation between painty and hexanal.

Inhibitor effects on beef flavor

In a preliminary report, St. Angelo et al. (1988) showed by chemical means a variety of chemical compounds could inhibit WOF in cooked beef. Of the many types used, some functioned as free-radical scavengers while others were chelating agents. Results showed many of the antioxidants at higher concentrations were effective inhibitors of WOF. In that preliminary study, there was no attempt to determine dose response for each additive nor was there an attempt to evaluate sensory properties of the experimental food samples, since many of the compounds were not on the "Generally Regarded As Safe" (GRAS) list.

Therefore, to determine if there were a difference in flavor in meat treated with the two types of antioxidants, *freshly* cooked ground beef patties containing compounds of each type were compared (Table 1). These patties had been marinated raw overnight at 4°C. Standards, which contained no additives and were made fresh the morning of the assay, had a CBB value of 6.4; PTY and CBD values were 0.0 and 0.1, respectively. The controls which contained 1 mL water, had the CBB value lowered to 5.4, whereas the PTY and CBD values increased slightly. Most of the experimental samples had flavor profiles similar to the water control. The samples with sodium ascorbate, which can function as a free radical or oxygen scavenger, electron donor, promoter of browning (Porter, 1980) or

Table 1—Effect of antioxidants on sensory properties of freshly cooked beef patties

Compound[a]	Cooked beef brothy[b]	Painty[b]	Cardboard[b]
Standard (no additives)	6.4 ± 0.2	0.0 ± 0.0	0.6 ± 0.1
Control (+ 1 mL water)	5.4 ± 0.3	0.2 ± 0.1	0.4 ± 0.2
Chelators			
Carrageenan-911	4.9 ± 0.4	0.5 ± 0.3	0.5 ± 0.2
Carrageenan-44[c] (2000 ppm)	5.5 ± 0.5	0.8 ± 0.4	0.8 ± 0.4
DTPA	6.2 ± 0.2	0.0	0.1 ± 0.9
Sodium citrate	5.8 ± 0.1	0.3 ± 0.3	0.1 ± 0.9
Sodium EDTA	6.3 ± 0.2	0.0	0.0
Sodium phytate (2000)	5.5 ± 0.0	0.2 ± 0.1	0.6 ± 0.2
Sodium pyrophosphate	6.1 ± 0.3	0.1 ± 0.1	0.5 ± 0.4
Sodium triphosphate	5.6 ± 0.4	0.1 ± 0.1	0.3 ± 0.2
Ascorbate-2-phosphate	5.9 ± 0.2	0.2 ± 0.2	0.4 ± 0.2
Ascorbate-2-triphosphate	5.5 ± 0.3	0.4 ± 0.2	0.6 ± 0.1
Free radical scavengers			
Maltol	5.1 ± 0.4	0.3 ± 0.2	0.3 ± 0.2
Maltol (250)	5.2 ± 0.3	0.1 ± 0.1	0.6 ± 0.6
Propyl gallate	5.3 ± 0.4	0.3 ± 0.3	0.7 ± 0.2
Rosemary	5.6 ± 0.2	0.0 ± 0.0	0.2 ± 0.2
Sodium ascorbate (1000)	6.5 ± 0.3	0.0 ± 0.0	0.1 ± 0.1
Tenox 20a (TBHQ/cit. acid)	5.6 ± 0.3	0.3 ± 0.2	0.4 ± 0.0
TBHQ	5.0 ± 0.4	0.2 ± 0.1	0.2 ± 0.1
Tenox 4 (BHA/BHT)	5.5 ± 0.3	0.1 ± 0.1	0.1 ± 0.1
α-Tocopherol acetate (250)	5.6 ± 0.3	0.5 ± 0.4	0.7 ± 0.4

[a] 500 ppm
[b] Mean flavor intensity scores ± s.e.m. (Meilgaard et al. 1987); 0 = not detectable
[c] Formulation contained pyrophosphate

Table 2—Effect of temperature on cooked beef patties[a] containing chelators and free radical scavengers after storage of 2 days

Compound[b]	Hexanal[c] −20°C	Hexanal[c] 4°C	TBARS[d] ± S.D.[e] −20°C	TBARS[d] ± S.D.[e] 4°C
Control (+ 1 mL water)	5.7	116	1.75 ± 0.03	7.79 ± 0.21
Chelators				
Carrageenan-911	4.0	177	4.78 ± 0.32	10.98 ± 0.01
Carrageenan-44 (2000 ppm)		124		5.91 ± 0.31
DTPA	0.3	0.2	1.01 ± 0.13	0.60 ± 0.04
Sodium citrate	6.1	99	2.43 ± 0.04	6.06 ± 0.02
Sodium EDTA		1.8		1.36 ± 0.63
Sodium phytate (2000)		29		2.19 ± 0.06
Sodium pyrophosphate		36		6.15 ± 0.16
Sodium tripolyphosphate	6.0	173	1.84 ± 0.02	6.86 ± 0.03
Ascorbate-2-monophosphate	1.5	258	1.40 ± 0.05	6.28 ± 0.05
Ascorbate-2-triphosphate	0.2	210	1.81 ± 0.15	8.91 ± 0.03
Ascorbate-2-mono-,di-,tri-,tetra-Phosphate (7,25,63,5)	1.3	123	1.23 ± 0.6	6.51 ± 0.05
Free radical scavengers				
Maltol	0.6	6	1.91 ± 0.04	1.85 ± 0.01
Maltol (250)	0.9	23	1.22 ± 0.02	2.44 ± 0.86
Propyl gallate	0.2	0.1	1.17 ± 0.09	1.03 ± 0.02
Sodium ascorbate (1000)		5		1.17 ± 0.04
Rosemary-W	2.4	86	1.07 ± 0.02	2.88 ± 0.05
Rosemary-O	0.6	19	1.23 ± 0.04	2.04 ± 0.01
Tenox 4 (BHA/BHT)	0.1	0.1	1.07 ± 0.15	0.69 ± 0.10
Tenox 20A (TBHQ/cit. acid)	0.2	0.7	1.51 ± 0.04	1.44 ± 0.01

[a] 80 g each
[b] Conc. 500 ppm
[c] Integrator area counts × 0.001
[d] mg malonaldehyde/kg ground meat
[e] Standard deviation

a weak chelator (Martell, 1982), has the highest CBB of the additives tested; TBHQ samples had the lowest. Propyl gallate, like many other trihydric phenols, can also chelate metals (Porter, 1980). One of the carrageenans was included with the chelators since this particular formulation contained sodium pyrophosphate and calcium monophosphate. In those patties, the PTY and CBD flavor notes were more intense. The same results were observed for patties containing tocopherol acetate, and ascorbate-2-triphosphate.

In general, the results implied that no appreciable differences in flavor notes among freshly cooked patties treated with additives and control samples. However, some additives contributed flavors additional to those listed on the ballot. For example, DTPA and propyl gallate added a burnt flavor; sodium citrate and Tenox 20A contributed a cowy/grainy flavor; sodium pyrophosphate, sodium ascorbate, the ascorbyl phosphates, TBHQ, EDTA and rosemary-W caused a more metallic/astringent mouthfeel; carrageenan-44 added cowy/grainy flavor and astringent mouthfeel, maltol (250 and 500 ppm) resulted in astringent/metallic mouthfeel and fruity/floral flavor. Consequently, even though concentration of these compounds did not appreciably decrease intensity of the CBB flavor, nor drastically increase intensities of PTY and CBD off-flavors, they contributed to other off-flavors.

Effects of storage temperature

Effect of storage at −20°C and 4°C on cooked beef patties containing both types additives and stored 2 days is shown in Table 2. The hexanal content of the controls increased at 4°C indicating those samples developed WOF. These data correlated well with TBARS values, which also increased for frozen samples compared to those stored at 4°C. The most effective inhibitors among the chelators were EDTA, DTPA, and sodium pyrophosphate. STP appeared to be ineffective in our system, as indicated by increases in hexanal and TBARS. The ascorbate/phosphate derivatives paralleled the STP. The carrageenan-44, which contained pyrophosphate, was more effective than STP. All of the free radical scavengers seemed to be effective inhibitors. Perhaps the phosphates were not as active as the free radical scavengers because the phosphates were more heat labile. In a previous study, many of these compounds appeared more effective when added to ground beef after cooking (St. Angelo et al., 1988). Greene (1969) previously reported polyphosphates were ineffective in fresh meats because they probably were hydrolyzed by phosphatases, but polyphosphates inhibited WOF in cooked meat. Smith et al. (1984) has also shown STP injected into raw beef, cooked and stored 3 days was ineffective in preventing WOF. Extracts from the natural spice, rosemary, had inhibitor activity, but a water formulation was not as effective as an oil-based formulation, or some other compounds tested. Rosemary contains many compounds with antioxidant properties. Four of these, rosmaridiphenol, rosmariquinone, rosmanol and carnosol, were identified as phenolic type compounds (Houlihan and Ho, 1985), which probably function as free radical scavengers similar to BHA, BHT, or TBHQ.

Sensory and chemical evaluation of cooked patties

The sensory and chemical evaluation of cooked beef patties, which contained chelators and free radical scavengers, stored 2 days at 4°C is shown in Table 3. These compounds were selected primarily because of their potential as inhibitors of WOF. The freshly cooked control patties, marinated overnight at 4°C, showed a decrease in CBB intensity, and an increase in the intensities of PTY, CBD, and TBARS over the frozen standard patties. These data suggest WOF was starting to develop in the raw ground beef patties during overnight storage. When compared to freshly cooked control, the 2-day stored controls showed greater decrease in CBB and greater increases in PTY, CBD, hexanal, and TBARS, all of which are indicative of WOF. Of samples with chelators added, those containing EDTA had lowest hexanal and TBARS values indicating WOF development. This inhibition occurred at both EDTA concentrations (125 and 500 ppm). Also, the CBB intensity was at the normal range and the intensity of the WOF descriptors, PTY and CBD, were not detectable. STP, α-tocopherol acetate (TA), and sodium citrate were ineffective at these concentrations. Tocopherol was also not effective (data not shown). Roozen (1987) reported low antioxidant activity in beef for TA and reported that was due to it being oxidized by Fe^{+3}. Patties that contained sodium pyrophosphate at the higher concentration had lower hexanal and lower TBARS than patties that contained lower concentrations. All of these samples had lower hexanal and TBARS values than the 2-day control. However,

Table 3—Effect of antioxidants on sensory and chemical properties of cooked beef patties stored 2 days, 4°C.

Compound (ppm)		Cooked beef[a] brothy	Painty[a]	Cardboardy[a]	Hexanal[b]	TBARS[c]
Standard[d]		6.4	0.1	0.1	16	1.99
s.e.m. (±)[e]		0.0	0.0	0.0	4	0.20
Control (+1 mL water)[f]						
Fresh cooked		5.7	0.3	0.5	12	4.51
s.e.m. (±)		0.1	0.0	0.0	2	0.07
2 Days WOF		4.9	1.7	1.7	82	6.97
s.e.m. (±)		0.1	0.1	0.1	10	0.57
Chelators						
Sodium citrate	(500)	5.3	1.5	1.6	81	5.42
Sodium EDTA	(125)	5.8	0.2	0.4	5	1.34
Sodium EDTA	(500)	5.6	0.2	0.4	0.2	0.04
Sodium phytate	(500)	5.6	0.9	0.9	19	1.00
Sodium pyrophosphate	(125)	5.2	1.2	2.0	127	4.91
Sodium pyrophosphate	(250)	5.1	1.0	1.2	76	3.55
Sodium pyrophosphate	(500)	4.7	1.4	1.4	33	3.35
Sodium triphosphate	(500)	4.7	1.8	1.9	112	5.43
Sodium triphosphate	(750)	5.2	1.0	1.0	40	6.30
Sodium triphosphate	(1000)	5.2	1.2	1.0	111	6.60
Free radical scavengers						
Ascorbyl palmitate	(500)	5.2	0.6	0.9	31	3.48
Maltol	(250)	5.6	0.3	0.8	29	2.14
Propyl gallate	(125)	6.0	0.1	0.2	0.1	1.52
Propyl gallate	(250)	6.0	0.1	0.2	0.1	1.30
Propyl gallate	(500)	5.8	0.6	0.7	0.06	0.74
Sodium ascorbate	(250)	5.9	1.2	1.4	52	6.55
Sodium ascorbate	(500)	5.5	0.7	1.2	14	1.47
Sodium ascorbate	(1000)	5.9	0.1	0.2	1	1.86
Tenox 4 (BHA/BHT)	(125)	5.9	0.2	0.4	0.1	1.86
Tenox 4	(250)	5.7	0.1	0.5	0.1	5.64
Tenox 4	(250)	5.5	0.5	0.6	0.1	0.99
Tenox 20A TBHQ/citric acid,	(125)	6.0	0.2	0.3	0.4	1.62
Tenox 20A	(250)	5.9	0.1	0.4	0.2	5.64
Tenox 20A	(500)	5.8	0.2	0.6	0.1	1.10
TBHQ	(125)	5.7	0.1	0.3	0.1	0.01
TBHQ	(250)	6.3	0.0	0.2	0.1	0.07
α-Tocopherol acetate	(500)	3.8	3.7	2.6	138	7.10

[a] Mean flavor intensities scores (Meilgaard et al. 1987); 0 = not detectable
[b] Integrator area count × 0.001
[c] mg malonaldehyde/kg ground meat
[d] Kept frozen, 3 days, then cooked and assayed; n = 16
[e] Standard error of the mean
[f] n = 13

Table 4—Effect of rosemary and sodium alginate on sensory and chemical properties of cooked beef patties stored (for 0 days/end 2 days) at 4°C

Compound (ppm)	Cooked beef broothy[a]	Painty[a]	Cardboardy[a]	Hexanal[b]	TBARS[c]
Control (+1 mL H_2O, n = 13)	5.65/4.85	0.29/1.71	0.50/1.72	12/82	4.51/6.97
Rosemary-W[d] (125 ppm)	5.60/5.30	0.01/0.94	0.31/1.50	1/73	1.09/3.64
Rosemary-W (250)	5.19/5.15	0.55/1.20	0.54/1.25	2/38	0.80/2.35
Rosemary-W (375)	5.30/5.00	0.31/1.10	0.41/1.60	1/51	0.90/2.19
Rosemary-W (500)	4.15/3.95	0.80/1.10	0.95/1.50	1/19	1.37/3.10
Rosemary-O[e] (50)	5.70/5.30	0.22/1.60	0.60/1.60	1/52	4.44/7.32
Rosemary-O (100)	5.70/5.50	0.20/0.64	0.30/0.89	2/38	2.40/4.79
Sodium alginate (500)	5.38/4.52	0.31/1.82	0.53/1.62	1/126	1.29/4.79
Sodium alginate (2000)[f]	5.20/5.30	0.60/0.70	0.80/0.80	1/47	1.22/3.30
Sodium alginate (5000)[g]	5.20/4.40	0.20/1.10	0.91/1.50	2/21	1.19/4.24
Sodium alginate (8000)[h]	3.97/4.54	0.23/0.33	0.84/0.88	4/15	4.14/3.26

[a] Mean flavor intensity scores (Meilgaard et al., 1987); 0 = not detectable
[b] Integrator area count × 0.001
[c] mg malonaldehyde/kg ground meat
[d] Water based
[e] Oil based
[f] Contains $CaCO_3$ (0.04%) and GDL (0.06%)
[g] Contains $CaCO_3$ (0.09%) and GDL (0.15%)
[h] Contains $CaCO_3$ (0.15%) and GDL (0.25%)

by increasing to the higher concentration of sodium pyrophosphate (500 ppm) the CBB intensity reduced to a value lower than the 2-day control, which indicated the desired beefy flavor character notes were diminished. Of the chelators containing polyphosphate, sodium phytate (sodium inositol hexaphosphate) was the most effective inhibitor as judged by low hexanal and TBARS values. Moreover, the intensity value for CBB remained acceptable (similar to the fresh cooked control), whereas the off-flavor notes or WOF descriptors were reduced by one-half when compared to the 2-day old WOF control. Since sodium phytate is naturally occurring, found abundantly in oilseeds, further investigation of its potential use to prevent WOF may be justified.

The data also indicate free radical scavenger compounds were effective in inhibiting PTY and CBD flavor and in retaining the high intensity of the CBB flavor. Propyl gallate (which is known to chelate metals), the Tenox formulations, and TBHQ were all very effective. PTY, CBD, hexanal and TBARS values remained low when compared to untreated samples, whereas the CBB flavor was retained at an intensity similar to fresh cooked beef.

Free radical scavengers had greater flavor preservation ef-

fects then did chelators. Thus, antioxidants that function as free radical scavengers should be more acceptable as additives for preventing WOF formation and retaining desirable CBB characteristics than the chelators. These data also suggest that free radical chemistry plays a very important role in WOF development.

Effects of rosemary and sodium alginate on MFD

Patties containing different concentrations of two formulations of rosemary oleoresin were evaluated for sensory and chemical values in freshly cooked (zero days storage) and in cooked patties at 4° C (stored 2 days) (Table 4). In the control patties, the hexanal and TBARS values were very low, but upon storage, those values increased. The sensory marker CBB decreased with storage, whereas the WOF sensory markers, PTY and CBD, increased. When rosemary-W was added to the patties, as concentration of rosemary increased from 125 ppm to 500 ppm, hexanal and TBARS decreased in both fresh and stored patties, which suggested lipid oxidation was being suppressed. However, CBB also decreased as the concentration increased. These results were observed in both the freshly cooked and stored patties. In fact, at 500 ppm in stored patties, which had the greated decrease in lipid oxidation, the CBB value dropped the most, 3.95. At the lower concentrations, 250 and 375 ppm, the CBB values for stored patties were above 5, whereas the hexanal and TBARS were lower than the control, or the sample treated with 125 ppm. The WOF descriptors, PTY and CBD increased with concentration in the freshly cooked patties, but these two flavor notes were fairly stabilized at slightly higher intensities in the stored patties. The intensity values of CBD were higher than those of PTY. These data suggested that perhaps while rosemary was protecting the patties from lipid oxidation, the effect was not as great on sulfur containing degradation products, which contribute to the CBD flavor note (Vercellotti et al., 1989).

In freshly cooked patties treated with rosemary type 0 at 50 ppm, hexanal contents were very low, 1000 counts; in the 2-day patties, hexanal count increased 52-fold. This increase was lower than the 2-day control, which had a 70-fold increase after storage. At the 100 ppm level, hexanal increased only 36-fold. TBARS values paralleled those of hexanal. At 100 ppm the panel rated the CBB intensity very high, whereas the PTY and CBD intensities received low scores. Consequently, rosemary-O appeared more effective than the water-based formulation.

In similar experiments, the effect of sodium alginate at concentrations ranging from 500 to 8000 ppm with varying binder levels were examined as inhibitors of WOF. The binder levels were 0.05 for 500 ppm, 0.3 for 2000 ppm, 0.74 for 5000 ppm, and 1.2 for 8000 ppm. These calculations were based on those reported by Clarke et al. (1988). In general, as concentration and binder levels increased in the stored patties, the chemical indicators, i.e., hexanal and TBARS values, decreased. Sensory evaluation of stored patties indicated that samples treated with 2000 ppm alginate, a binder level of 0.3, would be most acceptable of the treated samples. Those stored patties had a CBB value of 5.30, a PTY value of 0.70 and a CBD value of 0.80, all very acceptable. At the highest concentration, 8000 ppm, hexanal counts were down to a low of 15,000, TBARS was 3.26, the PTY score decreased to a low of 0.33, and the CBD was 0.88. These data indicated lipid oxidation was being reduced, but the CBB intensity decreased below 5, which suggested loss in the desirable CBB flavor. Previous reports (Means et al., 1987; Clarke et al., 1988) showed that alginate/calcium/GDL could serve as effective binders in restructured beef steaks. However, alginate treated steaks had lower flavor scores than those products without (Means et al., 1987). Also, binder levels greater than 0.57% resulted in higher cook yield and greater cooked product bind scores than those products without binders (Clarke et al., 1988). In our study, patties containing higher binder levels, 0.74 and 1.12, received lower CBB intensity scores.

CONCLUSIONS

Results indicated chelators and free radical scavengers could inhibit WOF in beef. However, intensity of desirable CBB flavor note, was reduced in 2-day stored patties treated with some of those compounds. Inhibitors that showed the smallest reduction in the CBB appeared to be those that act as free radical scavengers. Patties containing the more effective inhibitors were those that had the lowest PTY and CBD values and the highest CBB values. Perhaps the free radical scavengers were serving two functions, they retarded/inhibited lipid oxidation, as well as protein degradation. Free radical chemistry obviously played a major role in the complex WOF process. At low concentrations, rosemary, type O, appeared to be an effective inhibitor of WOF. Sodium alginate at 0.2% and binder level of 0.3 appeared to be the most effective alginate formulation tested. Finally, these studies showed a comprehensive sensory evaluation of experimental samples should be included with chemical or instrumental analyses when evaluating compounds as potential WOF inhibitors. For this purpose, a trained sensory panel is essential.

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Determination of Connective-Tissue Components in Beef Using Simultaneous Equations Based on Amino-Acid Analyses

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ABSTRACT

Collagen, elastin and residual actomyosin in connective tissue of some lower grades of beef were determined from the contents of hydroxyproline, valine and glutamic acid, using three simultaneous equations based on amounts of these amino acids found in the pure proteins. The complete amino-acid profiles of the connective tissues agreed with the calculated protein compositions. Connective tissues were removed from the meat by dissection, and sequential saline extractions removed sarcoplasmic and myofibrillar proteins. Glycoproteins were removed by extraction with half-saturated calcium hydroxide; collagen, by autoclaving. Changes in composition after extraction or autoclaving were confirmed by histological investigations.

INTRODUCTION

IN RESEARCH on muscle meat the need frequently arises for a simple way to determine the main protein components. Histological staining and microscopy have indicated variability in the ratios of such components. Forrest et al. (1975) identified ground substance, collagen, elastin, and reticulin. Bailey and Sims (1977) found Collagens I, III and IV in the connective tissue of meats, and immunospecific methods have indicated that reticulin may be identifiable as Type III collagen (Gay, 1978). Light and Champion (1984, 1985) found collagens IV and V in perimysium and endomysium muscle fibers. Partridge (1962) and Mellon et al. (1967) have isolated and determined the amino acid content of elastin from beef ligamentum nuchae. The ground substance contained glycoproteins consisting of protein complexes with chondroitin sulfate and with hyaluronic acid (Merkel, 1978). Sacks et al. (1988) selectively degraded the protein and polysaccharide components in muscle connective tissue and determined their effects on the viscoelasticity of muscle.

Zarkadas et al. (1988) proposed protein analysis of connective tissue by determining unique amino acids with special chromatographic columns. Our objective was to describe a simpler method of analysis, requiring only a conventional amino-acid analyzer. Dissolution of soluble proteins from isolated connective tissues of chuck and round left the insoluble residue of actomyosin, collagen and elastin, which make up 96+% of the connective tissue, prior to autoclaving. All the hydroxyproline, valine, and glutamic acid found were presumed to come from residual actomyosin, collagen or elastin. We could therefore obtain three simultaneous equations using amino acids found in the three unknown proteins actomyosin, collagen and elastin. The various components—glycoprotein, collagen, and elastin—were compared by histological examination before and after extraction.

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MATERIALS & METHODS

A FREEZE-DRIED center corium split from a limed beef hide prepared by the method described by Komanowsky et al. (1974) was used for reference collagen. Rabbit actomyosin and bovine neck ligament elastin were obtained from Sigma Chemical Corporation. The rabbit actomyosin was dialyzed to remove glycerol and potassium chloride before analysis.

Isolation of connective-tissue samples

The connective tissues used in these studies were carefully removed as epimysium, perimysium and endomysium from beef chuck and round beef cuts from eight animals at a local abattoir.

Epimysium, a sheath-like structure which surrounds each muscle, was removed manually from the surface. Both thick and thin pieces were studied because of obvious differences in texture and appearance. Perimysium, which is contiguous with epimysium and surrounds each fascicle of muscle fibers, was carefully removed from the chuck area of a single cut of beef. It was considered as a multicomponent sample since it contains endomysium, because of the manner by which it was obtained. Endomysium, which is contiguous with the perimysium and surrounds each muscle fiber within the fascicle, was carefully removed from chuck under a dissecting microscope.

All isolated connective tissues were first soaked in deionized water to remove water-soluble material or blood. Connective tissues were subjected to extensive extractions of a chloroform/methanol mixture (3:1, v/v) to dissolve associated fat. Connective tissues were cycled through several changes of deionized water to remove all traces of solvent and blotted dry with paper towels prior to moisture determination and salt extraction.

Salt extractions

After lipid extraction, separate portions of connective tissue or meat samples in the wet blotted state were weighed, and dry weights were calculated from moisture determinations (*vide infra*). The samples were extracted with increasing molarities of potassium chloride (0.15M, 0.45M, 0.75M and 1.00M) for 2 hr each at 25°C. This removed sarcoplasmic proteins, soluble proteins including soluble collagen, and some myofibrillar proteins. The salt-extracted connective tissue was washed thoroughly with deionized water before lime extraction. Before the salt extracts were analyzed, the wash solutions and extracts were combined and dialyzed extensively to remove potassium chloride. The protein material remaining after dialysis was evaporated to dryness using a rotary evaporator prior to hydrolysis and amino-acid analysis.

Glycoprotein extraction

Connective tissue present after potassium chloride extraction and washing was extracted with half-saturated calcium hydroxide solution for 24 hr to remove glycoproteins (Eastoe and Eastoe, 1954). The residue from this extraction was washed with deionized water until washings were neutral, dried under vacuum, ground with a Wiley mill, and redried under vacuum prior to hydrolysis and amino-acid analysis. The same procedures were applied to chopped intact pieces of chuck and round beef.

After glycoprotein extraction, one set of samples was autoclaved for four 45-min periods in deionized water at 121°C and 15 psig (Partridge 1962). Extracts were evaporated to dryness, residues were air dried and ground with the Wiley mill. All samples were vacuum dried prior to amino-acid analyses.

Table 1—Amino-acid compositions of principal proteins in connective tissue

Amino acids	Collagen g/g	Elastin g/g	Actomyosin[a] g/g
Hydroxyproline	0.1129	0.0138	------
Aspartic Acid	0.0583	0.0093	0.1007
Threonine	0.0181	0.0103	0.0500
Serine	0.0313	0.0092	0.0407
Glutamic Acid	0.1016	0.0254	0.1916
Proline	0.1383	0.1378	0.0303
Glycine	0.2155	0.2173	0.0279
Alanine	0.0809	0.1947	0.0556
Cystine	------	------	0.0206
Valine	0.0234	0.1596	0.0482
Methionine	0.0084	0.0012	0.0362
Isoleucine	0.0147	0.0331	0.0519
Leucine	0.0302	0.0815	0.0876
Tyrosine	0.0081	0.0173	0.0409
Phenylanine	0.0214	0.0535	0.0420
Isodesmosine	------	0.0041	------
Desmosine	------	0.0100	------
Histidine	0.0071	0.0017	0.0228
Hydroxylysine	0.0106	------	------
Lysine	0.0362	0.0071	0.0983
Arginine	0.0829	0.0127	0.0690
	100g AA/100g AA residues		

[a] Beef actomysin calculated on basis of 69% myosin and 31% actin.

Amino acid analysis

Protein samples were hydrolyzed with constant boiling hydrochloric acid under nitrogen for 24 hr. We found no loss of amino acids under these conditions. The acid was evaporated, and the samples were made up to volume in 0.1 N HCl. Analyses were done with a 119CL Beckman amino-acid analyzer at 60°C, using a 180-min single column (Fauconnet and Rochemont, 1978). All analyses were done in duplicate.

Moisture determination

An aliquot of the connective-tissue sample was weighed accurately, dried in a vacuum oven 48 hr at 50°C at less than 5 torr, and reweighed. The sample was then ground using a semimicro Wiley mill and passed through a #10 mesh screen. It was redried in the vacuum oven and weighed prior to hydrolysis and amino-acid analysis.

Histology

Connective tissue samples taken before and after the extractions with potassium chloride and calcium hydroxide and after autoclaving were fixed in 10% neutral formalin and prepared for histological evaluation. Sections were cut on a Spencer freezing microtome at 30 to 50 μm. The following stains were used for identification of specific elements: Weigert for elastin, Van Gieson (McClung, 1950) or picro-Sirius red F3BA (Sweat et al., 1964) for collagen and muscle, and Sudan IV or Oil Red O (Lillie, 1965) for fat identification and distribution. Toluidine blue (Johnson, 1968), a metachromatic stain, was used for identification of glycoproteins. Photomicrographs were made with a Zeiss Photomicroscope.

RESULTS & DISCUSSION

Amino-acid analyses of the principal proteins are shown in Table 1. Because purified beef actomyosin was unavailable, amounts of amino acids in beef actomyosin in Table 1 were calculated from our determination of rabbit actomyosin, using data of Bodwell and McClain (1978). Since the amino-acid profiles of actomyosin, collagen, and elastin differed markedly, the amounts of the three most variable amino acids could be used in three simultaneous equations to estimate the levels of these three proteins:

$$\begin{aligned} \text{Wt-\% Hydroxyproline} &= 0.1129C + 0.0138E + 0.0A \\ \text{Wt-\% Valine} &= 0.0234C + 0.1596E + 0.0482A \\ \text{Wt-\% Glutamic acid} &= 0.1016C + 0.0254E + 0.1916A \end{aligned} \quad (1)$$

Collagen has a large amount of hydroxyproline (11.29%, weight basis); elastin has little; and actomyosin has none. Similarly, elastin has a high valine content (15.96%) and actomyosin has a high glutamic acid content (19.16%). In the calculations the weight percentages of actomyosin (A), collagen (C), and elastin (E) in connective-tissue samples after all solubilized constituents had been removed were determined from Equation 1 and normalized so that they sum to 100%. Desmosine and isodesmosine were two amino acids found in small quantities in elastins from *ligamentum nuchae* and aortas (Partridge et al., 1963; Miller et al., 1964). Although their determination is possible, they were not used to determine elastin because of the small amounts present.

Table 2—Amino-acid composition of dissected connective tissue and chopped whole beef. Values calculated from protein (collagen, elastin, actomyosin) compositions determined here were compared with direct experimental values

	Connective tissue from chuck %		Chopped chuck roast %		Connective tissue from round roast %		Chopped round roast %	
	Calculated	Experimental	Calculated	Experimental	Calculated	Experimental	Calculated	Experimental
Hydroxyproline	5.714	5.681	0.721	0.698	6.299	6.194	1.946	1.884
Aspartic Acid	6.799	7.601	9.252	9.568	5.710	6.482	8.330	9.162
Threonine	2.916	2.993	4.569	4.543	2.395	2.695	4.032	4.158
Serine	3.196	3.493	3.812	3.850	2.844	3.104	3.553	3.667
Glutamic Acid	12.570	12.496	17.600	17.050	10.551	10.377	15.796	15.293
Proline	9.728	8.457	4.319	3.763	11.235	10.347	5.993	3.982
Glycine	14.467	13.001	3.952	4.374	17.104	15.184	7.968	7.053
Alanine	8.623	7.186	6.585	6.118	10.116	9.421	7.585	6.660
Cystine	------	------	------	------	0.049	------	------	------
Valine	5.063	5.035	5.388	5.220	6.083	5.982	5.730	5.547
Methionine	1.800	1.818	3.311	3.034	1.340	1.447	2.772	2.795
Isoleucine	3.129	3.414	4.862	5.027	2.785	3.139	4.382	4.822
Leucine	5.863	6.261	8.390	8.826	5.576	6.043	7.774	8.408
Tyrosine	2.173	2.468	3.756	4.003	1.807	2.195	3.296	3.639
Phenylanine	3.340	3.414	4.157	4.450	3.373	3.687	4.008	4.288
Isodesmosine	------	------	------	------	0.957	0.262	------	------
Desmosine	------	------	------	------	0.232	0.288	------	------
Histidine	1.232	1.657	2.058	2.607	0.961	1.274	1.785	2.214
Hydroxylysine	------	------	------	------	------	------	0.167	0.211
Lysine	5.588	6.012	8.902	9.568	4.430	4.731	7.783	8.624
Arginine	6.844	8.362	6.621	7.554	6.336	6.626	6.463	7.655
Protein	Collagen 49.01% Elastin 13.11 Actomyosin 37.88		Collagen 5.61% Elastin 6.35 Actomyosin 88.04		Collagen 52.96% Elastin 23.12 Actomyosin 23.92		Collagen 15.81% Elastin 11.68 Actomyosin 72.51	
Correlation	1.045		0.9980		0.9984		0.9936	

Table 3—Correlations obtained between the calculated amino-acid profile of connective tissue (with actomyosin) from chuck roast (Table 2) and the profile calculated for this connective tissue contaminated with various fractions of albumin

Albumin fraction	Correlation
0.240	0.98332
0.220	0.98605
0.200	0.98853
0.180	0.99076
0.160	0.99274
0.140	0.99448
0.120	0.99597
0.100	0.99722
0.080	0.99823
0.060	0.99901

Table 4—Amino-acid composition of protein extracts

Amino acid	g/100g protein Combined salt extracts	Glycoprotein extract
Methionine sulfoxide	0.21	0.35
Hydroxyproline	N.D.[a]	N.D.
Aspartic acid	9.78	10.48
Threonine	4.51	4.05
Serine	4.09	4.18
Glutamic acid	17.12	15.33
Proline	4.58	4.33
Glycine	4.21	3.80
Alanine	6.58	5.44
Cystine	1.41	0.11
Valine	5.88	5.66
Methionine	1.55	1.89
Isoleucine	3.64	3.86
Leucine	8.97	9.01
Tyrosine	3.13	3.63
Phenylanine	4.35	5.16
Glucosamine	N.D.	2.04
Galactosamine	N.D.	1.84
Histidine	2.83	3.73
Hydroxylysine	N.D.	N.D.
Lysine	10.87	9.69
Arginine	5.77	5.44

[a] N.D. = Not detected levels are below 0.3 mg/500 mg protein.

Table 5—Comparison of proteins in various connective tissues

Sample analyzed	% Actomyosin	% Collagen	% Elastin
Perimysium (multicomponent sample)	28.8	58.2	13.0
Thin epimysium	18.8	73.8	7.4
Thick epimysium	27.4	61.7	10.9
Endomysium	79.5	20.5	0

Equation 1 was used to estimate relative amounts of collagen, elastin and actomyosin in connective tissue and whole beef. These calculated values of and their respective amino-acid profiles (Table 1) were used to calculate amino acid contents of the tissues listed in Table 2.

The correlations between the calculated and experimental amino-acid profiles of the samples appear at the bottom of Table 2. These are defined as

$$\frac{\Sigma\, a_i\, b_i}{(\Sigma\, a_i^2)^{1/2}\,(\Sigma\, b_i^2)^{1/2}}$$

where a_i = calculated amount of amino acid i and b_i = determined amount. The correlation can vary from 1 (perfect agreement) to 0 (peptide with only one amino acid correlated with one that does not contain that amino acid). The significance of these correlations is shown in Table 3. The calculated amino-acid profile for connective tissue from chuck (Table 2) correlated with profiles calculated for various mixtures of that connective tissue composition with albumin. Values of the correlations for connective tissue from chuck and from round roast, 1.040 and 0.9984 respectively, correspond to levels of contaminant albumin that were too low to detect. From tables similar to Table 3 for the chopped meat samples, we found the correlations for chopped chuck and chopped round, which had been drastically extracted, but from which connective tissue had not been dissected, corresponded to 14% and 20% albumin, respectively. That was because albumin and the apparent major protein actomyosin had relatively similar amino-acid profiles. Therefore the residue from extraction of this meat that was neither collagen nor elastin was not pure actomyosin, but contained other proteins as well. This type correlation analysis would be necessary for confidence in results of this method especially if connective tissue had not been obtained by dissection.

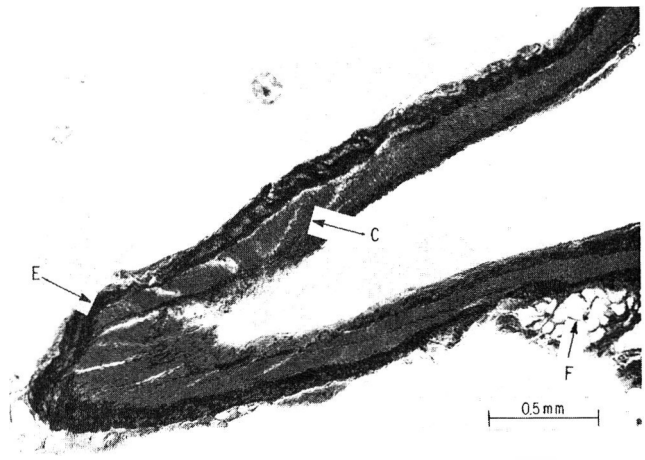

Fig. 1—Epimysium, showing collagen (C), elastin (E), and fat cells (F). Stained with Weigert and Van Gieson stains.

The results of sequential salt extractions of dissected connective tissue from chuck showed only 0.76% removal of starting material, indicating excellent isolation from the meat. The calcium hydroxide extraction removed 2.83%, the amount expected from the glycoprotein content. Glucosamine and galactosamine, each about 2% on a weight basis, were present in hydrolysates of the glycoprotein fraction, as expected from their presence in the glycosaminoglycan moiety of some glycoproteins. Amino acid analyses of the extracts are listed in Table 4. Collagen and probably elastin were absent, since hydroxyproline was not detected in the extracts. The amino-acid profiles of these lime extracts resembled that of the mucopolysaccharide extracted by Eastoe and Eastoe (1954). However, the lysine contents of our samples were twice those found in their analysis.

Table 5 shows protein content of the different types of connective tissues studied. Analyses showed that the dissected endomysium was probably highly contaminated with actomyosin; nevertheless the endomysium would seem to contribute little to toughness since it has no elastin, and a significantly lower amount of collagen was found in it than in the perimysium or in the epimysium. Under the microscope, muscle fibers were not evident in this tissue.

The presence of elastin in the epimysium and perimysium was shown by amino-acid analyses and histological procedures. Microscopy showed major differences in distribution of elastin in these tissues. It was uneven and random in the epimysium, less random and chiefly in the arterial systems of the perimysium, and essentially absent from the endomysium (Fig. 1, 2 and 3). The metachromatic effects of staining connective tissue with toluidine blue, indicating presence of glycoproteins associated with connective tissue, is shown in Fig. 4. Muscle tissue is shown at the top and bottom of the figure. After removal of the glycoproteins by the calcium hydroxide treatment, the metachromatic effect of toluidine blue is lessened, and this is also shown by the amino-acid analysis of the hydrolyzed alkaline extract (Table 4).

The fate of the connective tissues during autoclaving may be related to the cooking of meat. Table 6 shows that all the collagen was solubilized during the sequential autoclavings.

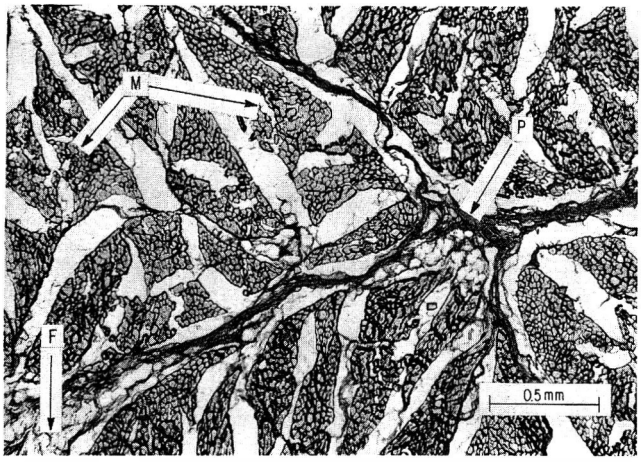

Fig. 2—Cross-section of meat from chuck, showing perimysium (P), muscle (M), and fat cells (F). Stained with picro-Sirius red.

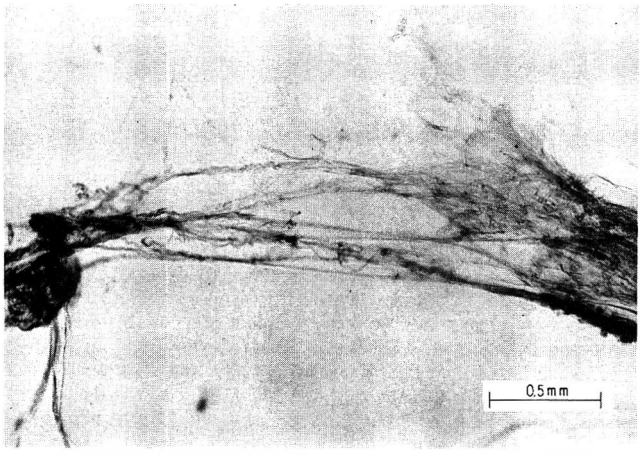

Fig. 3—Endomysium teased from muscle cells. Stained with Weigert and Van Gieson stains.

Most was removed during the first autoclaving, which extracted most of the heat soluble material, 43.7% as compared to 19.3% total for the second, third, and fourth extracts. Elastin in connective tissues was most resistant to this type heat treatment.

Meat with a high connective-tissue content is often treated with enzymes and other chemicals to degrade or soften these tissues and make the meat more palatable. The fractionation procedures and method described here help determine amounts of actomyosin, collagen, and elastin in connective-tissue samples. This should be useful in confirming feasibility and specificity of experimental treatments to selectivity remove or degrade specific connective tissue components.

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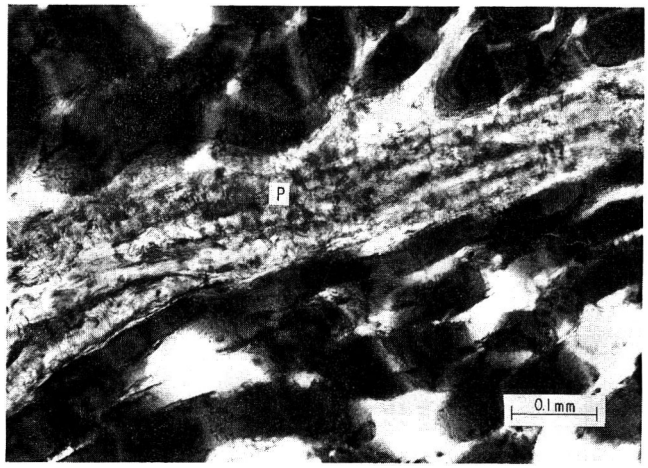

Fig. 4—Cross-section of meat showing perimysium (P) stained with Toluidine blue to demonstrate the presence of glycoproteins.

Table 6—Heat soluble proteins from autoclaved chuck connective tissue

Autoclave cycles	Proteins in extract (%)			Weight (%) of starting material
	Collagen	Elastin	Actomyosin	
1	95.1	0	4.9	43.7
2	89.0	1.1	9.9	15.1
3	46.1	5.0	48.9	2.9
4	14.9	8.3	76.8	1.3
Final residue	0	68.3	31.4	37.1

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Reference to brand or firm does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Effect of an Edible Collagen Film Overwrap on Exudation and Lipid Oxidation in Beef Round Steak

M.M. FAROUK, J.F. PRICE, and A.M. SALIH

ABSTRACT

Beef round steak when wrapped with an edible collagen film (Coffi®) exhibited much less fluid exudate ($P<0.05$) than unwrapped controls. This was true in refrigerated and frozen/thawed samples with standard retail (permeable film overwrap) or vacuum (pouch) packaging. Vacuum packaging produced more exudate and a less bright red color than retail packaging. Coffi® had no significant effect on color or lipid oxidation by TBA. Objective measures of color and exudate quantity highly correlated with panel scores for the same parameters (r values of 0.91 and 0.95). TBA values were lower ($P<0.05$) in refrigerated vacuum-packaged than in retail-wrapped samples. The impact of vacuum on TBA was less apparent in frozen samples.

INTRODUCTION

EXUDATION is the presence of surface juices in cut meat as a result of changes in water-holding capacity of muscle proteins (Lawrie, 1979; Briskey and Kauffman, 1978; Hamm, 1960). Lawrie (1979) referred to exudation of fluid from unfrozen meat as "weep" and the fluid that exudes from frozen/thawed meat as "drip." Accumulation of exudate in retail packages is unattractive to consumers, leads to loss of weight and value to wholesalers, promotes bacterial spoilage and often results in tough consistency and strawy taste (Taylor, 1982; Lawrie, 1979; Forrest et al., 1975; Hamm, 1960). Fahmy et al. (1981) reported "drip" contains an appreciable amount of minerals, proteins, vitamins and some flavor compounds. Fahmy and El-Kady (1984) found that drip in buffalo and camel meat contained a greater proportion of saturated fatty acids and a lower percentage total unsaturated fatty acids than the muscles from which the drip was collected.

Factors affecting water-holding capacity of meat include pH, metals, species of animal, sex, grade, breeding condition and treatment of animals before slaughter and postmortem changes. Wladyka and Dawson (1968) showed drip in chicken was higher in light than in dark meat. Amount of drip in cut meat is largely dependent on sample thickness, surface to volume ratio, orientation of cut surface with respect to muscle fiber axis and prevalence of large blood vessels (Lawrie, 1979; Khan and Lentz, 1977). Taylor (1972) established the clear anatomical distribution of drip loss with chop, chump and leg cuts having the highest amount of drip. Khan and Lenz (1977) reported slow freezing resulted in increased losses of drip and its constituents in comparison with fast freezing. Penny (1974) stated that pre-rigor freezing resulted in higher drip loss than post-rigor freezing. Very short (<5 min) and very long (>2000 min) thawing times produce greater than average drip losses (James et al., 1984).

Little has been reported on ways of minimizing exudation. However, some suggested measures include: rapid cooling of the carcass before onset of rigor mortis (Dann, 1972; Taylor, 1972), preconditioning of meat at 10°C for 2 or 5 days (Penny, 1974), packaging to prevent shrink of packaging material too tightly onto surface of meat and maintaining proper temperature control within display cabinets (Malton and James, 1983).

Coffi® is an edible collagen film (Brechteen Co.), widely used as a coating for specialty smoked meats (hams) and roasted meat products which are heat-processed in elastic stretch netting or coarse stockinette. This circumvents difficult netting removal and avoids resultant undesirable appearance and tearing of meat.

The purpose of this study was to determine if exudation in beef round steaks could be reduced, without affecting their color and TBA number, by using an edible collagen film (Coffi®) overwrap.

MATERIAL & METHODS

Sample preparation

Fresh beef top rounds, USDA Choice or USDA Select, were purchased from Ada Beef Co. (Ada, MI) on Fridays and held over the weekend at 4°C until processed on Monday. The top rounds were trimmed of visible fat, and 56 steaks averaging 254g, 11 cm × 10 cm and 1.6 cm thick were cut from the semi-membranous muscle. The steaks were divided into four treatment groups: (1) steaks were each separately put into a 25 cm × 37 cm vacuum bag (high barrier bags, Koch Equipment Co., Kansas City, MO) and a vacuum of 54.6 cm was pulled with a Koch model AGW Multivac; (2) steaks were wrapped with 65 cm × 75 cm piece of Coffi® film supplied by Brechteen Co. (Mt. Clemens, MI) before vacuum packaging; (3) individual steaks were tray-packed (25 type polystyrene foam) with polyvinyl chloride (PVC) film having O_2 permeability of 800–1000 mL/24 hr; (4) steaks were wrapped with the same size Coffi® as in group 1 and tray-packed with PVC as in 3. Each group was further divided into two subgroups of seven steaks each. Seven steaks in each subgroup were slowly frozen at the rate of 0.19°C/hr (initial freezing rate) and 0.75°C/hr (final freezing rate) and stored at −25°C for a week. The remaining seven steaks in each subgroup were stored for a week under simulated retail conditions at 1°C and 12 hr of fluorescent light (cool white, 110 to 135 ft-c) 45 cm away from the packaged steaks. Four steaks in each treatment subgroup were weighed before and after wrapping with film and again after one week storage. The frozen steaks were thawed overnight at 4°C before samples were taken for color measurements, sensory evaluations and thiobarbituric (TBA) acid analysis. This process was repeated twice.

Objective measurements

TBA values of four steaks in each treatment subgroup were determined using an extraction method (Salih et al., 1987). Color of steaks was measured using a Hunter color difference meter (Model D25-2) standardized with a pink tile (L = 68.8, a = 23.3 and b = 9.4). Measurements were made through the packaging film on two steaks from each group and averaged. The amount of exudate was measured by weighing the steaks before packaging and storage and again at the end of storage after removal of packages. Exudate was defined as the difference in weight.

Sensory evaluation

Twelve panelists familiar with exudation and meat color were chosen from the faculty, graduate students and workers in the Meat Laboratory. Two steaks from each treatment group were evaluated for exudate amount and color acceptability on day zero and again after one week of storage. The panel evaluation was conducted in the Meat Laboratory of Michigan State University. Panelists were given ques-

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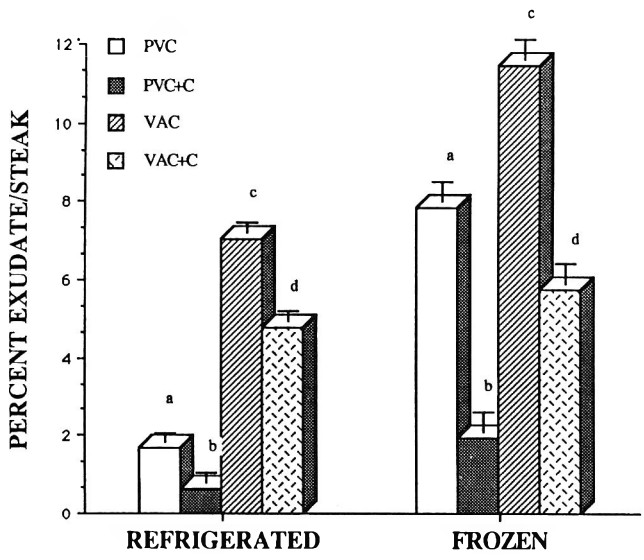

Fig. 1—Effect of storage condition and treatment on exudate amount in beef round steak. Bars under same condition bearing same letters are not significantly different (P ≥ 0.05). PVC = Tray pack with polyvinyl chloride (oxygen permeable overwrap or standard retail wrap. PVC + C = Individual steaks wrapped with Coffi® and tray pack with PVC. VAC = Vacuum pouch packaging. VAC + C = Vacuum packaging of individual steaks wrapped in Coffi®.

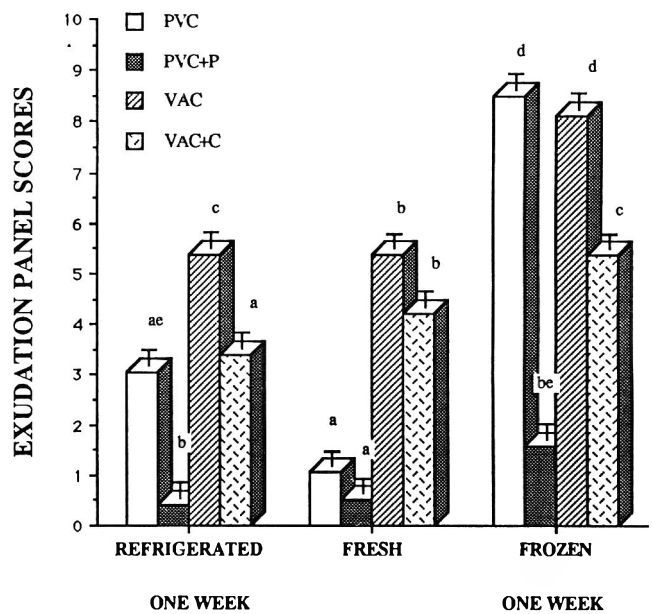

Fig. 2—Panel scores of exudation in steaks refrigerated at 1°C or frozen at −25°C. Bars under same condition bearing same letters not significantly different (P ≥ 0.05). Scale: 0 = no exudate, 10 = large amount of exudate. See Fig. 1 caption for treatment codes.

tionnaires based on descriptive analysis with scaling (Larmond, 1977). Panelists used a 10 cm line scale. Absence of exudate was indicated at 0 and large amount of exudate was indicated at 10; extremely desirable = 0 and extremely undesirable = 10 in reference to steak color.

Statistical analysis

The experimental design was a randomized block. Data were analyzed using an analysis of variance (ANOVA) statistical program, Microstat (Ecosoft, 1984). The means were compared using Tukey's test.

RESULTS & DISCUSSIONS

Effect of Coffi® film on exudation in beef round steaks

The effect of Coffi® film and storage conditions on amount of exudate is shown in Fig. 1. Samples that were slowly frozen and thawed had higher exudate than the refrigerated samples, irrespective of packaging methods (Fig. 1). Khan and Lentz (1977) reported slow freezing resulted in increased loss of drip compared to fast freezing. Anon and Calvelo (1980) found that amount of exudate varied in relation to freezing time. Results showed that among the four different treatments, steaks wrapped with Coffi® film and packaged with polyvinyl chloride had a significantly lower ($P < 0.05$) amount of exudate than those that were not wrapped with Coffi® film, whether frozen or refrigerated. Steaks wrapped with Coffi® before vacuum packaging also had significantly ($P < 0.05$) less exudate than corresponding vacuum-packaged steaks without Coffi®.

Included among the factors that affect extent of drip in meat are those which determine the extent to which fluid once formed will drain from meat (Lawrie, 1979). Therefore, the presence of a film in close contact to the surface of the steaks could reduce the amount of exudate. Much of the exudate could have been absorbed by the Coffi® film. In comparison of vacuum-packaged and tray-packaged steaks, those samples that were vacuum-packaged had significantly higher ($P < 0.05$) amounts of exudate than the corresponding steaks not vacuum-packaged. This observation is in support of Rizvi (1981), who reported vacuum packaging of meat resulted in about 0.1-2% of fluid per unit weight being lost from the surface of meat as "purge".

The panel scores for exudation (Fig. 2) correlated well with the amount of exudate measured objectively ($r = 0.91$). No significant differences ($P > 0.05$) were found between panel scores for freshly prepared samples wrapped with Coffi® film and those not wrapped with the film for vacuum-packed and tray-packed steaks. There was, however, a significant difference (P 0.05) in amount of exudate observed on day zero between samples that were vacuum-packaged and those tray-packaged with PVC. This is due to "purge" caused by vacuum packaging (Rizvi, 1981). Panel scores of samples refrigerated or frozen 1 wk (Fig. 2) indicated those samples wrapped with Coffi® film before packaging had significantly ($P < 0.05$) less exudate than the corresponding ones not Coffi® wrapped. Samples frozen and thawed had significantly ($P < 0.05$) more exudate than the refrigerated samples. The panel results were closely correlated ($r = 0.91$) with objective measurements of exudate amount.

According to Fahmy and El Kady (1984), Fahmy et al. (1981), Lawrie (1979) and Forrest et al. (1975), loss of exudate from meat leads to loss of nutrients and flavor. The lower amount of exudate among samples wrapped with Coffi® film (Fig. 1 and 2), therefore, meant that loss of nutrients, flavor and the detracting effect of visible exudate in retail packages was reduced. Exudate in retail packaged meat was reported to be unattractive to consumers (Taylor, 1972; Hamm, 1960).

Effect of Coffi® film on color of retail packaged steaks

Kropf (1980) and Westerberg (1971) stated that meat color was probably the single most important factor affecting retail marketability. Thus, in retail marketing, attempts to reduce exudate should avoid causing a color problem that would offset any advantage gained. Hunter color reflectance "a" measurements of fresh, refrigerated and frozen samples are shown in Fig. 3. The "a" values are emphasized because they are assumed to be indicators of redness and perceived color of meat. No significant difference ($P > 0.05$) existed in "a" values (redness) between samples wrapped with Coffi® and those that were not wrapped either on day zero or after storage 1 wk.

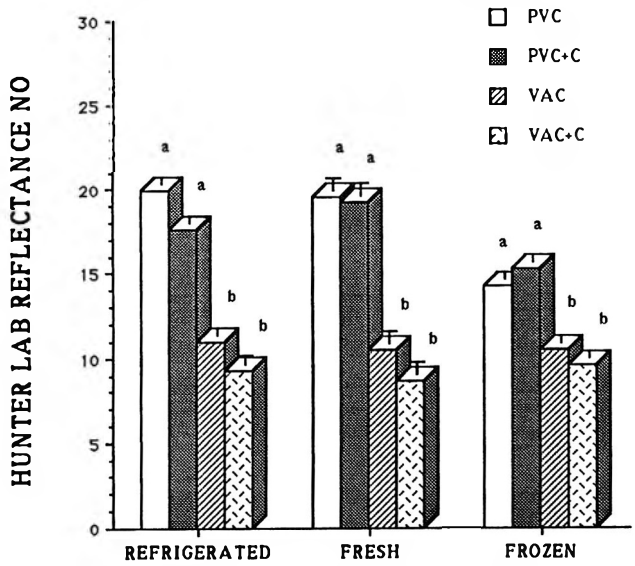

Fig. 3—Color reflectance measurements ("a" value) of freshly wrapped steaks and steaks refrigerated at 1°C or frozen at −25°C for 1 wk. Bars under same conditions bearing same letters not significantly different (P ≥ 0.05). See Fig. 1 caption for treatment codes.

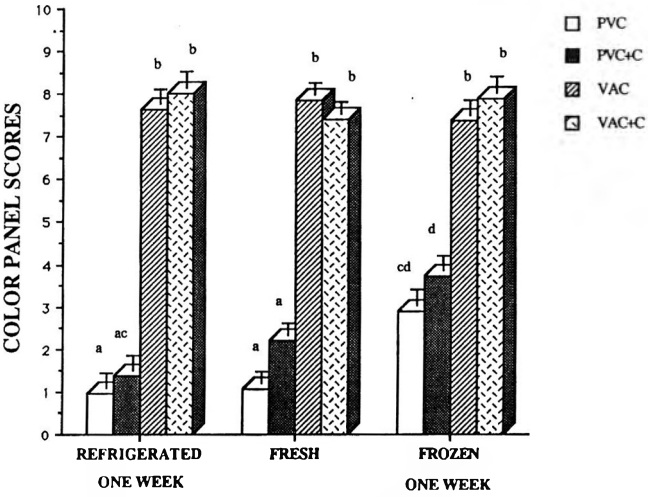

Fig. 4—Color panel scores of steaks refrigerated at 1°C or frozen at −25°C. Bars under same conditions bearing same letters not significantly different (P ≥ 0.05). Scale: 0 = extremely desirable, 10 = extremely undesirable. See Fig. 1 caption for treatment codes.

The only significant ($P < 0.05$) difference was between vacuum-packaged samples and nonvacuum-packaged. The difference was due to deoxygenation of the red oxymyoglobin to purple anoxic reduced myoglobin owing to absence of oxygen in vacuum packages. Similar observations were reported by Seideman et al. (1984) and Taylor (1982). The values of the color panel scores in Fig. 4 closely correlated with the color reflectance measurements. No significant difference ($P > 0.05$) was found between steaks wrapped with Coffi® and those that were not, at zero day or after 1 wk storage for refrigerated or frozen samples. Panel scores differed ($P < 0.05$) between vacuum-packaged samples and those packaged with PVC. This was observed by Taylor (1982) and Hood and Riordan (1973) and attributed to existence of the purple color in vacuum-packaged meats. A strong correlation ($r = 0.95$) was found between objective (Hunter reflectance "a" values) and subjective (panel scores) measurements of color. Wrapping steaks with

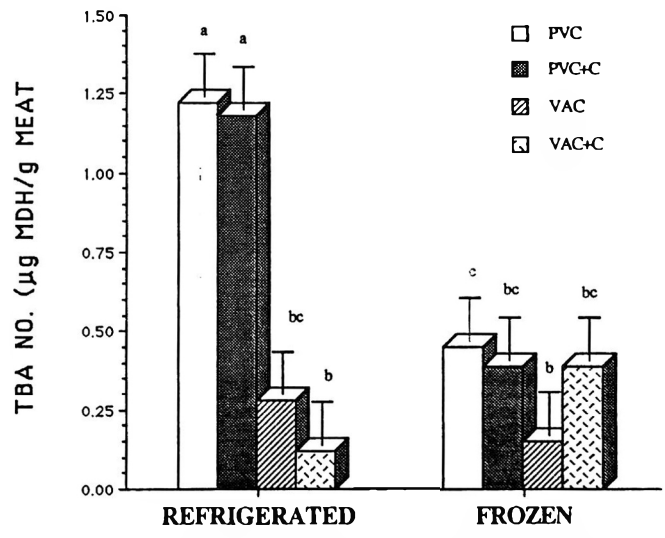

Fig. 5—TBA no. of steaks refrigerated at 1°C or frozen at −25°C for 1 wk. Bars under same conditions bearing same letters not significantly different (P ≥ 0.05). See Fig. 1 caption for treatment codes. MDH = Malonaldehyde

Coffi® to reduce exudation apparently would not affect color acceptability of the steaks by consumers.

TBA results

TBA values of steaks frozen 1 wk were generally very low (Fig. 5). Steaks overwrapped with PVC and held refrigerated 1 wk had significantly higher TBA values than any frozen samples or vacuum-packaged refrigerated samples. Vacuum packaging did not seem as critical in reducing TBA in short time frozen storage as in refrigerated storage. Chang et al. (1961) showed oxidation was slower in frozen meat than refrigerated meat. Booren et al. (1981) did not observe changes in TBA values in samples of sectioned and formed steaks after storage 91 days at −30°C. Miles et al. (1986) demonstrated that vacuum packaging lowered TBA values ($P < 0.01$) in restructured pork. Coffi® film did not affect ($P > 0.05$) TBA number in steaks.

In summary, exudation could be minimized without affecting color by wrapping steaks with Coffi® film. Vacuum packaging was not as beneficial in reducing lipid oxidation in short term frozen storage as in short term refrigerated storage (1°C). However, vacuum packaging caused more exudation compared to tray packaging with PVC, which resulted in more desirable color than vacuum packaging.

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Lipid Oxidation and Color Stability in Restructured Meat Systems during Frozen Storage

J.G. AKAMITTATH, C.J. BREKKE, and E.G. SCHANUS

ABSTRACT

Salt and combinations of salt with phosphates and antioxidants were investigated for their role in lipid oxidation and discoloration in restructured beef, pork, and turkey steaks during storage at −10°C for 16 wk, 8 wk and 8 wk, respectively. Lipid oxidation and discoloration occurred simultaneously in pork and turkey, but beef color loss occurred much earlier than did lipid oxidation. Phosphates were effective in inhibiting lipid oxidation in beef (4 wk), pork (8 wk) and turkey (6 wk). Tertiary butylhydroquinone inhibited lipid oxidation in pork and turkey steaks, but, overall, neither prevented discoloration. Results indicate discoloration and lipid oxidation are interrelated, and pigment oxidation may catalyze lipid oxidation.

INTRODUCTION

VALUE of some red meat, such as the beef chuck, or of dark poultry meat can be enhanced through restructuring techniques. Restructured meats are products that are partially or completely disassembled, then reformed into the same or different form. Restructuring facilitates accurate portion control, better compositional control, easier slicing and serving, and accurate prediction of cooked yields and servings (Pearson and Tauber, 1984) and, above all, the processor can design the meat products to fit consumers' specific needs.

Several serious problems, namely, color instability and fat oxidation, are encountered in restructured meats. Variable color patterns of restructured meat, especially beef products, continue to trouble processors, and color instability is a prime limitation to acceptance of restructured meat products in the retail trade (Secrist, 1982).

Previous studies suggested lipid and myoglobin oxidation are closely related, but no conclusive evidence has been reported to demonstrate if myoglobin catalyses lipid oxidation or vice versa (Govindarajan and Hultin, 1977). Although such studies have been done with ground beef and model systems, the additional steps involved in manufacture of restructured meat products, such as mixing, tempering, reforming and freezing, present a different reaction system compared to systems reported in previous studies. Thus, research is needed in the areas of lipid and pigment oxidation to understand interrelationships involved. This will help resolve the significant problems in acceptability of restructured meat products.

A compounding problem has been that studies on color stability and lipid oxidation in restructured meats have involved different species (Marriott et al., 1987), formulations (Simunovic et al., 1985), processing protocols (Trout and Schmidt, 1984; Means and Schmidt, 1986), storage temperatures and days of storage. Moreover, several different objective methods have been used to evaluate pigment stability, which has made comparing previous studies even more complex (Stewart et al., 1965; van den Oord and Wesdorp, 1971; Krzywicki, 1979; Booren et al., 1981).

The major objective of our study was to evaluate lipid oxidation and pigment stability of restructured steaks from beef, pork and turkey using comparable methodology within a single study. Within this objective, evaluation was included of the effect of salt alone or in combination with sodium pyro/polyphosphate, ascorbyl palmitate plus tocopherol, and tertiary butylhydroquinone on lipid and myoglobin oxidation.

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MATERIALS & METHODS

Meat and additives

Fresh (within 1 week of slaughter) trim from USDA Choice beef plate and frozen (1 month at −32°C in oxygen-impermeable wrap) trim from an entire pork carcass (Washington State University Meat Science Laboratory), fresh cow round (Hill Meats, Pendelton, OR) and frozen (2 wk at −24°C in oxygen-impermeable wrap) hand-deboned thigh meat from young hen turkeys and turkey abdominal fat (Norbest, Salem, OR) were used in this study.

Formulation additives included Alberger Shur-Flo Salt and Curox 12LC food-grade antioxidant (ascorbyl palmitate + alpha-tocopherol) (Diamond Crystal Salt Co., St. Clair, MI), Brifsol 414, a commercial blend of sodium pyro/polyphosphate (BK- Ladenberg, Cresskill, NJ), and tertiary butyl hydroquinone (TBHQ) (Eastman Kodak Chemical Products, Newport, TN).

Treatments

Treatments were: C, control (no additives); S, salt (1.5%, w/w); SP, salt (1.5%) and sodium pyro/polyphosphate (0.3%, w/w); SAT, salt (1.5%) and ascorbyl palmitate/alpha tocopherol (0.02%, based on final fat content of the restructured steaks); ST, salt (1.5%) and tertiary butylhydroquinone (TBHQ) (0.02%, based on final fat content of the steaks). All treatments had ice incorporated in the formulation at 3% (w/w). Restructured beef steaks included C, S, SP and SAT, whereas pork and turkey resturctured steaks additionally had ST.

Steak Manufacture

Cow round, serving as the source of lean, was trimmed of bone, excess fat and connective tissue. Choice beef plate, the fat source in the formulation, and cow round were separately ground once through a 1.0 cm plate using a meat grinder (Butcher Boy A52.50H; Laser Manufacturing Co., Inc., Los Angeles, CA). Random samples of the ground meat were removed and analyzed for fat by the Modified Babcock Method (Koneicko, 1979). Calculated amounts of ground round (2.5% fat) and beef plate (42.0% fat) were weighed and combined to obtain 15% final fat in the restructured beef steaks. Ice and the treatment ingredients (salt, and/or phosphates, and/or antioxidants) were blended for 8 min in a ribbon-type food mixer (Leland Detroit Mfg. Co., Detroit, MI). The blended mix was stuffed (Frey 20 liter Electric-Hydraulic Stuffer (nonvacuum); Koch Supplies, Inc., Kansas City, MO) into 2.0 × 68.5 cm fibrous casing (Viskase Sales Corp., Chicago, IL) to form a log, and the logs were blast-frozen for 15–16 hr at −30°C. Frozen logs were sawed into 2.5 cm thick steaks. Each steak was wrapped in a meat freezer wrap (1 mil low-density polyethylene; Zell Freezer Film, 20-ZF; Zellerbach Paper Co., San Francisco, CA), and held at −10°C for up to 16 wk.

The process was repeated for pork and turkey. Fat content of the raw materials for pork and turkey was: pork lean trim (12.0%), pork fat trim (74.0%), turkey thigh meat (9.3%), and turkey abdominal fat (78.6%). Pork and turkey steaks were held at −10°C for up to 8 wk.

*Table 1—Proximate composition of restructured steaks**

Treatment	Species	Moisture (%)	Fat (%)	Protein (%)	Ash (%)
C	Beef	65.06	15.37	18.60^{a}	0.89^{a,d}
	Pork	64.18	15.54	19.35^{t}	0.93^{b,g}
	Turkey	65.26	15.74	17.26^{c}	0.85^{c,l}
S	Beef	66.62	14.71	17.41^{a}	2.26^{a,e}
	Pork	66.56	15.08	19.56^{t}	2.26^{a,h}
	Turkey	66.54	14.77	17.10^{a}	2.01^{b,m}
SP	Beef	66.90	15.47	18.52^{a,b}	2.46^{a,f}
	Pork	65.02	15.23	18.89^{t}	2.43^{b,j}
	Turkey	65.66	15.16	17.90^{a}	2.31^{c,n}
SAT	Beef	66.71	15.19	18.74^{a}	2.24^{a,e}
	Pork	64.19	14.76	19.32^{t}	2.25^{a,h}
	Turkey	65.71	15.22	17.67^{c}	2.11^{b,o}
ST	Beef	—	—	—	—
	Pork	63.73	15.49	19.02^{t}	2.12^{a,k}
	Turkey	65.75	14.94	16.87^{c}	2.09^{b,o}

* Means of three samples/treatment. (Control (C); salt—1.5% (S); salt—1.5% + phosphate — 0.3% (SP); salt—1.5% + ascorbyl palmitate/alpha-tocopherol—0.02% (SAT); salt—1.5% + tertiary butylhydroquinone—0.02% (ST)).

a-c Means with different superscripts in the same column, within a treatment, are significantly different ($P < 0.05$).

d-o Means with different superscripts in the same column for beef (d,e,f), pork (g,h,j,k) and turkey (l,m,n,o) are significantly different ($P < 0.05$).

*Table 2—Mean thiobarbituric acid values for restructured beef, pork, and turkey steaks**

Species	Storage (Weeks)	mg Malonaldehyde/kg meat C	S	SP	SAT
Beef	0	0.67^{afv}	0.96^{afw}	0.56^{afv}	1.09^{afw}
Pork	0	0.14^{bhv}	0.21^{bhv}	0.02^{bhv}	0.10^{bhv}
Turkey	0	0.49^{anv}	1.34^{cnw}	0.14^{bnx}	1.24^{anw}
Beef	1	0.62^{afv}	1.09^{afw}	0.50^{afv}	1.01^{afw}
Pork	1	0.16^{bhv}	0.55^{bjw}	0.06^{bhv}	0.35^{bjw}
Turkey	1	0.52^{anv}	2.00^{cow}	0.63^{aov}	2.26^{cox}
Beef	2	0.57^{afv}	1.10^{afw}	0.63^{afv}	1.10^{afw}
Pork	2	0.33^{bhjv}	0.95^{akw}	0.07^{bhx}	0.62^{bky}
Turkey	2	0.72^{anov}	2.37^{bpw}	0.99^{cpx}	2.69^{cpy}
Beef	3	0.64^{bfv}	0.96^{afw}	0.69^{afv}	1.09^{afw}
Pork	3	0.32^{ahjv}	1.21^{blw}	0.13^{bhv}	1.14^{alw}
Turkey	3	1.05^{cpv}	2.28^{cpw}	1.82^{cpx}	3.76^{bsy}
Beef	4	0.66^{afv}	1.09^{afw}	0.79^{afv}	1.20^{afw}
Pork	4	0.37^{bhjv}	1.59^{bmw}	0.43^{bjv}	1.58^{bmw}
Turkey	4	0.95^{copv}	3.12^{cqw}	2.02^{cqx}	3.18^{cqw}
Beef	6	0.60^{afv}	1.10^{afw}	1.05^{agw}	1.48^{agx}
Pork	6	0.53^{ajv}	1.93^{btw}	0.59^{bjv}	1.63^{amx}
Turkey	6	0.90^{bopv}	3.23^{cqw}	2.91^{crx}	3.47^{bry}

*Each value is the mean of three samples/treatment/species/week. (Control (C); salt - 1.5% (S); salt - 1.5% + phosphate - 0.3% (SP); salt - 1.5% + ascorbyl palmitate/alpha-tocopherol - 0.02% (SAT)).

a-c Means with different superscripts in the same column, within a storage time are significantly different ($P < 0.05$).

f-t Means with different superscripts in the same column for beef (f,g), pork (h-m,t), and turkey (n-s) are significantly different ($P < 0.05$).

v-y Means with different superscripts in the same row, for the same species, are significantly different ($P < 0.05$).

Analyses

Three steaks from each treatment were randomly selected at each sampling. For color analyses, triplicate measurements per steak were obtained, then averaged. Hunter Color scores ("a_L" and "L") were measured using a Hunter Color Difference Meter (Model D25) calibrated with a standard pink tile (#D 25 W825, L = 73.0, a_L = 10.8, b_L = 5.7). Final mean for that treatment was obtained by averaging means from each of the three steaks sampled.

The three steaks used for color evaluation were also used for chemical analyses. Proximate analysis for fat, protein, moisture and ash was performed in triplicate for all treatments as outlined by AOAC (1980). Oxidative rancidity was monitored using the 2-thiobarbituric acid (TBA) procedure of Salih et al. (1987).

Statistical analyses. Data were subjected to analysis of variance using a completely random design as described by Steel and Torrie (1960). Since every treatment was not assigned to each species, and time of storage varied for species, statistical analysis involved time and treatment common to the three species. In the case of treatments unique to a species, statistical comparisons were made for that treatment within the same species. Fisher's Least Significant Difference Test (Steel and Torrie, 1960) was used to determine differences between species, treatments and storage time when F values were significant. Pearson's Correlation Coefficient was calculated for TBA and Hunter "a_L" values of restructured beef, pork and turkey steaks. Homogeneity of the correlation coefficient (significant difference) was calculated as discussed in Steel and Torrie (1960).

RESULTS & DISCUSSION

COMPOSITIONAL ANALYSES (Table 1) of the restructured steaks for the three species revealed that, in comparison to the control, ash content significantly ($P<0.05$) increased with the salt and the combination of salt with phosphates or antioxidants, as was expected. No significant treatment differences among the three species for moisture and fat content were found. However, small but significant ($P < 0.05$) differences in protein were found among the three species for some treatments.

Oxidative rancidity

Effect of salt on lipid oxidation. Significant ($P < 0.05$) interactions of lipid oxidation with species, treatment and storage time were indicated by analysis of variance. For species, thiobarbituric acid analysis (TBA) revealed, overall, significant differences ($P < 0.05$) in lipid oxidation over the period of storage (Tables 2 and 3). Although the data in Tables 2 and 3 cover similar values, the least significant difference values differ for each table because they are based on different sets of treatments, species and storage periods as indicated in the Statistical Analyses section above. Although not rapid, turkey and pork control steaks showed a similar, slight, but significant, increase in TBA numbers compared to beef during 6 wk storage (Table 2). Also, turkey and pork salt-treated steaks showed a rapid and similar increase in TBA numbers compared to beef during 6 wk storage (Table 2). The rapid increase in TBA values for turkey and pork may be attributed to the greater ratio of unsaturated to saturated fatty acids normally associated with these species in comparison with beef (Allen and Foegeding, 1981).

Salt enhanced lipid oxidation in comparison to C for the three species (Table 2 and 3). Similar findings have been reported by Huffman et al. (1981) and Lamkey et al. (1986). The mode of action of salt is still unclear, but a possible explanation for the detrimental action of salt might involve water activity as influenced by the freezing conditions of our study. Water activity of the meat system could be altered due to freeze-concentration and salt effects, shifting to the range of intermediate moisture foods where lipid oxidation occurs at a rapid rate (Labuza, 1971). Another explanation could be the oxidation of myoglobin by salt via an anion-promoted autoxidation process as reported by Wallace et al. (1982) and Chu et al. (1987). Autoxidation of myoglobin can lead to formation of metmyoglobin (MMb) and hydrogen peroxide (H_2O_2) which can interact with each other to produce an activated molecule, "activated metmyoglobin — MMb-H_2O_2," that can initiate lipid oxidation (Harel and Kanner, 1985; Kanner and Harel, 1985).

Effect of pyro/polyphosphates on lipid oxidation. In our study, steaks containing sodium pyro/polyphosphates (SP) had significantly lower TBA values for beef through 4 wk and from pork through 8 and turkey 6 wk respectively, in comparison to S for the three species (Table 2 and 3). However, with time, the prooxidant effect of salt and other factors, such as activated MetMb-H_2O_2 and the presence of free metal ions, overcame the protective effect of phosphate.

*Table 3—Mean thiobarbituric acid values for restructured pork and turkey steaks**

Species	Storage (Weeks)	mg Malonaldehyde/kg meat				
		C	S	SP	SAT	ST
Pork	0	0.14^{ahv}	0.21^{ahw}	0.02^{ahv}	0.10^{ahv}	0.04^{ahv}
Turkey	0	0.49^{amv}	1.34^{bmw}	0.14^{amv}	1.24^{bmw}	0.37^{amv}
Pork	1	0.16^{ahv}	0.55^{ahw}	0.06^{ahv}	0.35^{ahjv}	0.10^{ahv}
Turkey	1	0.52^{bmv}	2.00^{bnw}	0.63^{bnv}	2.26^{bnw}	0.65^{bmnv}
Pork	2	0.33^{ahjv}	0.95^{ajw}	0.07^{ahv}	0.62^{ajv}	0.12^{ahv}
Turkey	2	0.72^{bmnv}	2.37^{bow}	1.00^{bov}	2.69^{bow}	0.92^{bnv}
Pork	3	0.32^{ahjv}	1.21^{ajw}	0.13^{ahjv}	1.14^{akw}	0.13^{ahv}
Turkey	3	1.05^{bnv}	2.28^{bow}	1.82^{bpx}	3.76^{bpry}	1.26^{bnov}
Pork	4	0.37^{ahjv}	1.59^{akw}	0.43^{ajv}	1.58^{alw}	0.20^{ahjv}
Turkey	4	0.95^{bnv}	3.12^{bpw}	2.02^{bpx}	3.18^{bqw}	1.56^{boy}
Pork	6	0.53^{ajv}	1.93^{akw}	0.59^{ajkv}	1.63^{alw}	0.29^{ahjv}
Turkey	6	0.90^{bnv}	3.23^{bpw}	2.91^{bqx}	3.47^{bqrw}	1.56^{boy}
Pork	8	0.63^{ajvw}	1.26^{ajx}	0.39^{akw}	1.03^{akx}	0.44^{ajv}
Turkey	8	0.92^{anv}	2.47^{box}	2.59^{bqx}	3.67^{bry}	1.44^{bow}

Each value is the mean of three samples/treatment/species/week. (Control (C); salt—1.5% (S); salt—1.5% + phosphate—0.3% (SP); salt—1.5% + ascorbyl palmitate/alpha-tocopherol—0.02% (SAT); salt—1.5% + tertiary butylhydroquinone—0.02% (ST)).

[a,b] Means with different superscripts in the same column, within a storage time, are significantly different ($P < 0.05$).

[h-q] Means with different superscripts in the same column for pork (h-l) and turkey (m-q) are significantly different ($P < 0.05$).

[v-y] Means with different superscripts in the same row, for the same species, are significantly different ($P < 0.05$).

Phosphates inhibit lipid oxidation either by acting as chelators of free metals (Timms and Watts, 1958) and/or by increasing pH (Miller et al., 1986). Recent studies by Kanner et al. (1988) indicate poultry meat (chicken or turkey) contains significant amounts of free catalytic iron, which may be responsible for the susceptibility of the muscle tissues to lipid oxidation. The concentration of free iron ions increases with storage time. Thus, the phosphate concentrations used in our study may have been effective for a limited period due to the chelation of free iron. As the concentration of free iron increased, as described by Kanner et al. (1988), the stoichiometric balance shifted, leaving an excess of iron ions with no free phosphate to chelate.

Moreover, the type of phosphate might also affect the nature of its effectiveness within a particular species. Lamkey et al. (1986) concluded from their studies on restructured beef steaks that salt increases the rate of rancidity development, whereas the addition of phosphate reduces the rate. However, studies on restructured beef steaks by Miller et al. (1986) and Chu et al. (1987) indicate phosphates do not impart beneficial action in inhibiting lipid oxidation, although the phosphate in each of these studies varied in composition and level of use.

Effect of antioxidants on lipid oxidation. Overall, ascorbyl palmitate plus alpha-tocopherol, in the presence of salt (SAT), did not inhibit lipid oxidation in the three species during their respective storage periods compared to S (Tables 2 and 3). Antioxidants such as ascorbates and tocopherol are concentration-dependent in their inhibitory activity. At low concentrations, ascorbates act as prooxidants (Sato and Hegarty, 1971; Roozen, 1987). The supplier of the ascorbyl palmitate/alpha tocopherol recommended a useage concentration of 0.1% of the fat content. However, the alpha-tocopherol and ascorbyl palmitate concentrations used in our study, 0.02%, were based on previous studies on restructured meats (Chastain et al., 1982; Miles et al., 1986). Miles et al. (1986) reported antioxidant treatments consisting of alpha-tocopherol and ascorbate were effective in controlling lipid oxidation in restructured pork, but the salt concentration in their study was 0.5%, and the temperature of storage was 4°C. They also reported salt and tocopherol treatments had greater TBA numbers than the other antioxidant treatments, and they suggested that tocopherols are variable in their effect, i.e., alpha-tocopherol may be less effective as an antioxidant than gamma-tocopherol. In our study, pork samples consistently had significantly ($P < 0.05$) lower TBA numbers (Table 3) for SAT treatment when compared to turkey. In addition, pork SAT steaks were also significantly lower in TBA values for 0 to 2 wk than beef SAT steaks, but, thereafter, pork steaks were not significantly different, overall, from beef steaks. Thus, there evidently exists a species effect on functioning of ascorbyl palmitate and tocopherol in restructured steaks during storage.

*Table 4—Mean Hunter "a_L" values for restructured pork and turkey steaks**

Species[2]	Storage (weeks)	C	S	SP	SAT
Beef	0	15.71^{afv}	16.33^{afv}	15.46^{afv}	15.97^{afv}
Pork	0	14.50^{amv}	14.38^{bmv}	14.67^{amv}	14.85^{amv}
Turkey	0	14.69^{aqv}	15.37^{aqv}	15.93^{aqv}	15.06^{aov}
Beef	1	14.30^{agv}	10.98^{agw}	11.43^{agw}	11.79^{agw}
Pork	1	13.68^{amv}	12.44^{anv}	13.44^{bmv}	12.12^{anv}
Turkey	1	13.25^{aqv}	8.34^{brw}	11.58^{arv}	8.20^{brw}
Beef	2	12.91^{agv}	10.16^{agw}	10.36^{agw}	10.43^{agw}
Pork	2	10.47^{bnv}	8.09^{bow}	10.41^{anv}	8.24^{bow}
Turkey	2	11.37^{arsv}	8.37^{brw}	8.48^{bsvw}	7.63^{brw}
Beef	3	12.83^{aghv}	9.88^{agw}	8.61^{abhw}	8.58^{ahw}
Pork	3	12.50^{anv}	7.33^{bopw}	10.32^{anv}	7.18^{bopw}
Turkey	3	10.02^{bsv}	7.70^{brsw}	7.88^{buw}	6.86^{brw}
Beef	4	12.31^{aghv}	7.91^{ahw}	8.06^{abhw}	8.78^{ahw}
Pork	4	11.70^{anv}	7.16^{aopw}	9.21^{anox}	7.00^{bopw}
Turkey	4	9.43^{bsv}	7.02^{arsw}	6.91^{buw}	6.88^{brw}
Beef	6	11.05^{ahv}	7.36^{ahw}	7.22^{ahw}	7.90^{ahw}
Pork	6	11.17^{anv}	5.69^{apw}	8.22^{aox}	6.06^{bpw}
Turkey	6	9.58^{asv}	6.35^{asw}	7.07^{aux}	6.54^{brx}

*Each value is the mean of three samples/treatment/species/week. (Control (C); salt—1.5% (S); salt—1.5% + phosphate—0.3% (SP); salt—1.5% + alpha-tocopherol/ascorbyl palm tate—0.02% (SAT)

[a-c] Means with different superscripts in the same column, within a storage time, are significantly different ($P < 0.05$).

[f-u] Means with different superscripts in the same column for beef (f-h), pork (m-p) and turkey (q-u) are significantly different ($P < 0.05$).

[v-y] Means with different superscripts in the same row for the same species are significantly different ($P < 0.05$).

Tertiary butylhydroquinone (TBHQ) inhibited lipid oxidation in restructured pork and turkey steaks compared to S (Table 3). These results showing the effectiveness of TBHQ as an antioxidant concur with the findings of Chastain et al. (1982) and Crackel et al. (1988). Pork ST steaks had TBA numbers less than pork C steaks, and turkey ST steaks had TBA values greater than turkey C steaks (Table 3) during 8 wk storage, again suggesting the effectiveness of antioxidants is species-specific.

Color stability. One-way analysis of variance performed on Hunter "a_L" values indicated significant differences ($P < 0.05$) due to species, treatment and storage time. As seen in Tables 4 and 5, all treatments within each species decreased in redness

*Table 5—Mean Hunter "a_L" values for restructured pork and turkey steaks**

Species	Storage (Weeks)	C	S	SP	SAT	ST
Pork	0	14.50^{ahv}	14.38^{ahv}	14.67^{ahv}	14.85^{ahv}	14.20^{ahv}
Turkey	0	14.69^{amv}	15.37^{amv}	15.93^{amv}	15.06^{amv}	15.99^{bmv}
Pork	1	13.68^{ahkv}	12.44^{ajv}	13.44^{ahv}	12.12^{aiv}	10.36^{ajw}
Turkey	1	13.25^{anv}	8.34^{bnw}	11.58^{bnx}	8.20^{bnw}	10.33^{any}
Pork	2	10.47^{ajv}	8.09^{akw}	10.41^{ajv}	8.24^{akw}	8.74^{akw}
Turkey	2	11.37^{aov}	8.37^{anw}	8.48^{aow}	7.63^{anox}	8.33^{aow}
Pork	3	12.50^{akv}	7.33^{akx}	10.32^{ajv}	7.18^{aklw}	7.97^{aklw}
Turkey	3	10.02^{bopv}	7.70^{anow}	7.88^{bpw}	6.86^{aow}	7.69^{bov}
Pork	4	11.70^{akv}	7.16^{akx}	9.21^{ajkw}	7.00^{aklx}	7.92^{aklx}
Turkey	4	9.43^{bpv}	7.02^{anow}	6.91^{bpqw}	6.88^{aow}	7.49^{aow}
Pork	6	11.17^{akv}	5.69^{alkx}	8.22^{aklw}	6.06^{alx}	8.11^{aklw}
Turkey	6	9.58^{bpv}	6.35^{aow}	7.07^{apqw}	6.54^{aow}	8.09^{aovw}
Pork	8	9.62^{alv}	5.36^{akx}	7.02^{alw}	5.59^{alw}	6.74^{alw}
Turkey	8	8.97^{bpv}	6.01^{aow}	6.16^{aqw}	7.04^{bow}	8.06^{bow}

*Each value is the mean of three samples/treatment/species/week. (Control (C); salt—1.5% (S); salt—1.5% + phosphate—0.3% (SP); salt—1.5% + ascorbyl palmitate/alpha-tocopherol—0.02% (SAT); salt—1.5% + tertiary butylhydroquinone—0.02% (ST)).

a,b Means with different superscripts in the same column, within a storage time, are significantly different ($P < 0.05$).

h-q Means with different superscripts in the same column for pork (h-l) and turkey (m-q) are significantly different ($P < 0.05$).

v-y Means with different superscripts in the same row, for the same species, are significantly different ($P < 0.05$).

*Table 6—Pearson's Correlations (R) and probabilities (P) of thiobarbituric acid (TBA) values and Hunter "a_L" values in restructured beef, pork and turkey steaks**

Species		Treatments C	S	SP	SAT	ST
Beef	(R)	−0.65^{a}	−0.54^{a}	−0.60^{a}	−0.50^{a}	
	(P)	0.0001	0.0017	0.0004	0.0042	
Pork	(R)	−0.66^{a}	−0.85^{b}	−0.84^{b}	−0.84^{b}	−0.55^{a}
	(P)	0.0010	0.0001	0.0001	0.0001	0.0092
Turkey	(R)	−0.66^{a}	−0.69^{a}	−0.87^{b}	−0.82^{b}	−0.68^{a}
	(P)	0.0009	0.0004	0.0001	0.0001	0.0006

*Correlation coefficients pooled over time. (Contro (C); salt—1.5% (S); salt—1.5% + phosphate—0.3% (SP); salt—1.5% + ascorbyl palmitate/alpha-tocopherol—0.02% (SAT); salt—1.5% + tertiary butylhydroquinone—0.02% (ST).

a,b Correlation coefficients with the same superscr pts within a column or a row are not significantly different ($P < 0.05$).

during storage. Based on Hunter values and informal visual observations, the rate of discoloration in S was found to be the greatest in turkey, followed by pork and then beef. Phosphates prevented pigment discoloration to some extent (3 wk) compared to S treatments (Table 5) in restructured pork steaks, but, overall, phosphates were not effective in protecting red color.

Most reported studies on restructured meat have dealt only with "a_L" values, since the consumer associates redness with acceptability of red meat. However, care must be taken in interpreting "a_L" values alone, since color measured by "a_L" values is part of the tristimulus coordinate system (L, a_L, b_L). That is, Hunter "a_L" scores are influenced by "L" and "b_L" values. Since the interest in our study was in redness of the steaks, "b_L" values were not determined. Unfortunately, due to inconsistency in "L" values (data not shown), relating "a_L" and "L" Hunter values was not feasible nor was comparing the three species. Informal visual observation of restructured steaks revealed that the steaks (all treatments) appeared darker with storage, but Hunter Colorimeter "L" values did not indicate differences.

Relationship between lipid oxidation and color stability. Correlation coefficients (Table 4) suggest an inverse relationship exists between lipid oxidation and pigment stability in restructured meats during frozen storage. The correlation, although slight, was significant ($P < 0.05$). Significance was partially influenced by the small number of values ($n = 30$) used to determine correlation coefficients.

Correlation coefficients for most treatments (S, SP, SAT) in beef were fairly low and significantly different (Table 6). A possible explanation is the initial lag (0-4 weeks) in TBA for beef steaks, i.e., discoloration occurred during this period, but there was no significant increase in TBA numbers for beef. Another reason could be that oxidative degradation was first perceived in pigments and only observed in lipids at later stages of oxidation, as reported by Torres et al. (1988). Since oxidation of myoglobin and oxymyoglobin to metymyoglobin (MMb) is a common process in intact red meat and restructured meat systems, we can reasonably assume the H_2O_2 produced endogenously and via autooxidation of myoglobin could activate MMb to form activated MMb-H_2O_2, that could catalyze lipid oxidation, as reported previously (Harel and Kanner, 1985; Kanner and Harel, 1985; Rhee et al., 1987). This may explain the initial discoloration (lag period), followed by lipid oxidation, seen in salt-containing beef steaks. The TBA test is possibly not as sensitive to oxidative changes in pigment oxidation as concluded by Torres et al. (1988). However, in our study, lipid oxidation and color loss occurred simultanelously in pork and turkey for S, SP and SAT treatments. This might be due to the smaller amounts of total pigment present in pork and turkey in comparison to beef, such that, for equal extent of pigment oxidation, actual color changes occured in pork and turkey sooner than in beef. Thus, with pork and turkey, rapid oxidation of pigments might have provided a pool of biological catalyst that initiated lipid oxidation in the relatively unsaturated fat system compared to beef.

Although TBHQ (ST) treatment in pork and turkey inhibited lipid oxidation, it did not prevent discoloration, accounting for the small and significantly different correlation values in comparison to the S treatment. TBHQ is an electron donor and deactivates the catalyst responsible for lipid oxidation. In our study, oxidation of meat pigments in pork and turkey possibly resulted in activated MMb-H_2O_2 that initiated lipid oxidation. Hydroperoxide radicals generated during the initiation phase were probably suppressed by the phenolic antioxidant TBHQ.

The storage temperature used in our study was −10°C (14°F), which is not normal commercial frozen storage temperature. This temperature was used to accelerate fat oxidation and color change, that is to decrease the storage time required for changes. However, storage temperatures of −11°C to 12°C favor oxidation of meat pigments (Zachariah and Satterlee, 1973), which may have influenced our results.

CONCLUSIONS

DISCOLORATION and lipid oxidation of restructured steaks are related. In beef, color degradation occurred much earlier in storage than did lipid oxidation, and the oxidized pigments may have catalyzed lipid oxidation. The delay in lipid oxidation was due to greater proportions of saturated to unsaturated fatty acids. The time required for initiation and propagation of

oxidized saturated fatty acids might have been longer than for unsaturated fatty acids. Small but important increases in lipid oxidation may have occurred, but been undetected due to lack of sensitivity of the TBA analysis. In comparison to beef, pork and turkey had a greater proportion of unsaturated to saturated fatty acids, and the unsaturated fatty acids were susceptible to lipid oxidation. That explained the simultaneous occurrence of lipid and pigment oxidation in pork and turkey. Better methods to measure lipid and myoglobin oxidation, and color loss inhibition to prevent pigment oxidation from influencing lipid changes, will lead to more definitive conclusions about cause-and-effects between lipid and myoglobin oxidation.

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Sodium and nitrite determination

No difference was seen in sodium or nitrite composition across treatment (Table 7). This corresponded with sensory panel data which showed no difference in saltiness across treatments and yield data which showed no differences in pump, massage, cook or overall yields across treatments.

CONCLUSIONS

THE ADDITION of high oleate oils to the diet of growing-finishing swine did not affect most sensory, textural, visual or chemical parameters. However, higher a percentage of taste panelists detected off-flavors in the canola oil treatment hams than all other treatments. Hams from swine of 10% safflower oil or sunflower oil were acceptable in all sensory, physical and chemical characteristics.

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Characteristics of Beef Batters as Influenced by Electrical Stimulation and Postmortem Salting Time

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ABSTRACT

Changes were evaluated in functional and textural properties of beef batters altered by mincing, postmortem salting time and electrical stimulation. Histological examinations showed raw batters from non-stimulated (NS) meat exhibited uniform fat dispersal throughout the protein matrix regardless of salting time; however, fat channeling, increased fat and gel-water release, lower hardness and gel strength along with reduced apparent viscosity were observed in electrically stimulated (ES) meat when salting was delayed. Changes in amounts of salt-soluble protein were not consistent. Effects of pH on water-holding capacity and myofibrillar swelling accounted for most changes observed in batters from ES muscles. However, increases in apparent viscosity and hardness may have been a consequence of rigor onset.

INTRODUCTION

EFFECTS OF SALT (NaCl) and pH on water-holding capacity (WHC) of muscle minces have been reviewed by Hamm (1977, 1981, 1986). Water-holding capacity was shown to be the primary element involved in production of stable meat batters rather than effects due to solubility of salt soluble myofibrillar proteins (Hamm, 1973, 1981). Salt increased hydration and swelling of meat proteins (Offer and Trinick, 1982) (increased WHC) by an anion "screening" effect which immobilized water and fixed fat in the meat batter when cooked (Hamm, 1960; Schut, 1976). Fat fixed in this protein matrix was physically immobilized with little released during cooking (Hamm, 1975). Prerigor meat, salted with over 1.8% NaCl, had greater WHC and better fat "emulsifying" properties than meat in the rigor or postrigor state (Hamm, 1975). These superior functional properties were lost when salting was delayed due to on-set of rigor mortis (Hamm, 1982).

The application of electrical current to beef carcasses to increase tenderness and reduce heat ring (Savell et al., 1978) may have deleterious effects on functional properties of meat used in manufacture of finely comminuted products (bologna and frankfurters). Electrical stimulation (ES) is widely recognized to hasten onset of rigor mortis by speeding ATP turnover, accelerating glycolysis and lowering pH (Fabiansson and Reutersward, 1985; Newbold and Small, 1985). Terrell et al. (1982) found slight differences in moisture content of beef frankfurters produced with meat from electrically stimulated beef carcasses when compared to those made from nonstimulated beef, but attributed these primarily to increased fat. They concluded there were no real advantages or disadvantages for using ES beef in manufacture of commercial frankfurters. Other researchers reported similar findings and concluded electrical stimulation does not affect the functional properties of postrigor meat (Hamm, 1986; Whiting et al., 1981). Conversely, Choi et al. (1984) indicated preblended hot-boned Triceps brachii (containing 3.0% NaCl) had higher emulsion capacity than preblends from cold-boned muscles, but emulsion capacity values were reduced when hot-boned muscles were electrically stimulated. In addition, batters made from electrically stimulated muscles (both hot- and cold-boned) had lower ($P<0.05$) thermal stability than those from nonstimulated muscles. In their model system, electrical stimulation had undesirable effects on functional characteristics of preblended meat resulting in lower emulsion capacity and less thermal stability. These effects were believed caused by changes in protein structure which decreased heat stability of the protein-fat matrix. This may have been caused by fast accumulation of lactic acid and rapid drop in pH in the ES muscles while the musculature was at a reasonably high temperature (Choi et al., 1984).

The maintenance of high WHC and functional properties of prerigor salted meat may also be influenced by electrical stimulation. Consequently, this study was conducted to demonstrate effects of salting prerigor beef muscles at five postmortem salting times (1, 4, 8, 12 and 24 hr) on the apparent viscosity, textural traits and thermal stability of beef batters.

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MATERIALS & METHODS

Slaughter and meat preparation

Three steers (737 kg avg live weight, ranging from US Select to US Standard) were slaughtered on separate days at the University of Kentucky Meats Laboratory. One side of each carcass was randomly chosen and electrically stimulated (ES) (550 volts; 2.2 amps; 70 one-sec pulses, one sec on, one sec off; followed five min later with 20 one-sec pulses) approximately 30 min after sticking (Grubbs, 1982). The remaining side was not electrically stimulated (NS). Muscles (Biceps femoris, Semimembranosus, Semitendinosus, Adductor, Gracilis, Gluteus medius) were removed within 1 hr postmortem, trimmed of subcutaneous and intermuscular fat and divided into five fractions randomly allotted to five postmortem salting times (i.e., 1, 4, 8, 12 and 24 h). Each fraction contained about 3 kg meat. At the appropriate time, muscles were minced (2.5 cm), salted (3.5% NaCl), mixed (5 min) in a Hobart vertical mixer (model A200D, on speed setting 1) to thoroughly distribute the salt, placed in plastic bags and refrigerated (5°C) until analyzed. The meat from each animal constituted a complete replication. Salted meat samples were composed of 21.0% crude protein, 4.4% ash, 73.2% water and 1.4% fat (by difference).

pH

The pH of the minced salted meat was determined immediately after salting at 1, 4, 8, 12 and 24 hr (denoted ESI and NSI) and after holding 48 hr (4°C) denoted (ES48 and NS48) by homogenizing 5 g muscle with four parts (w/w) buffered iodoacetate (pH 6.8) for 15 sec in a Waring Blendor (Bendall, 1978). Likewise, the pH of the slurries (apparent viscosity) was determined by combining 20g slurry with four parts distilled water. Iodoacetate was not used since glycolysis had been arrested by addition of NaCl. All measurements were made using a Radiometer pH meter equipped with combination electrode.

Apparent viscosity

Preliminary work indicated reproducible measurements of apparent viscosity could be made using a meat slurry containing 200% added water and ionic strength 0.55. Salted meat (68.3 g; 5°C) was placed in a Sears Counter Craft ™ food processor (Sears, Robuck and Co.) and combined with 133.4 mL chilled (5°C) 2.6% NaCl solution (w/v) and chopped 2 min at speed setting 5. The meat slurry was poured into a 250 mL beaker and allowed to stand 1 hr (to equilibrate to 24°C) before measuring apparent viscosity using a Brookfield Synchro-electric rotary viscometer (model: RVT; Brookfield Engineering Lab., Stoughton, MA) at 50 rpm with spindles 4 or 6 with guard

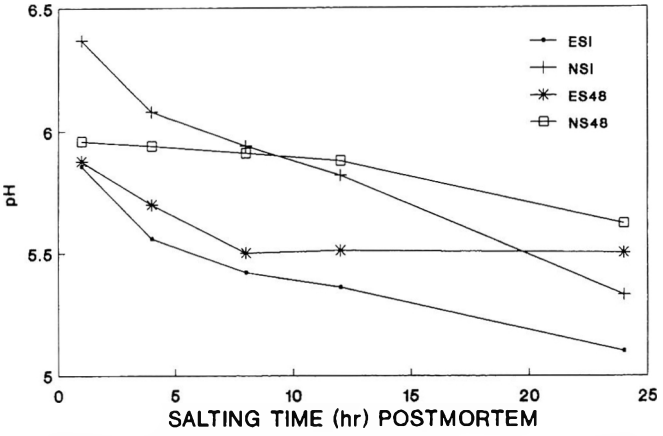

Fig. 1—LSMeans of the pH of beef muscles from electrically stimulated (ES) and nonstimulated (NS) carcasses minced and salted at various times postmortem (ESI, immediately after salting; NSI, immediately after salting; NS48, 48 hr after salting; ES48, 48 hr after salting.

removed and reported in centipoise (cp). Four determinations were made for each experimental unit.

Thermal stability

Salted meat (126g; 5°C), 10% ice (calculated on the salted meat weight) and 54g lard (Fischer Packing Co. Louisville, KY) were chopped in a Sears food processor according to the following schedule: (1) salted beef was chopped 30 sec, (2) ice and lard were added and the mixture chopped 30 sec, (3) the bowl was scraped and the mixture chopped again for 30 sec. Total chopping time was 1.5 min; final chopping temperature averaged 19°C. Although final chopping temperature was slightly higher than recommended for meat batters, it was chosen to (1) provide adequate disintegration of the muscle tissue, and (2) to stress the meat batter system to accent differences which might be caused by the various treatments. Three plastic centrifuge tubes (50 mL) per treatment were filled with 30 g meat batter, capped, centrifuged at 200 × g 10 min and cooked in 70°C water bath for 30 min (Whiting, 1987). The liquid contents of the tubes were immediately poured into 15 mL conical tipped glass graduated tubes and centrifuged at 200 × g 10 min. Cookout (fat, gel-liquid and solids) was read directly from the centrifuge tubes and recorded. The gel strength (g) required to penetrate the plug of cooked batter in the centrifuge tube was measured by the procedure of Whiting (1987) but modified by using a 4.76 mm threaded steel rod rather than a 6.35 mm rod. The threaded rod reduced lubrication effects of the released lard.

Texture measurements

Three additional 50 mL plastic centrifuge tubes were filled with 30 g batter and prepared as above for texture assessment using the General Foods Texture Profile analysis (Bourne, 1968). After cooking, the meat plug was removed from the tube, one end squared and two slices cut 13 mm thick using a scalpel. These slices were placed between two steel plates on an Instron Universal Testing Machine (model TM) and compresseed 50% of their original thickness at a cross head speed of 5 cm/min. Values for hardness (peak force in kg) were recorded.

Histological examination

Samples of batter produced for the cook stability test were removed for histological examination using light microscopy. Samples (ca. 5g) were frozen in liquid nitrogen, wrapped in aluminum foil and held frozen (−18°C) until sectioned. Samples were prepared as described by Lee (1985). Sections (12 μm) were cut using a freezing microtome, stained with oil red O and counterstained with Harris hematoxylin.

Salt-soluble protein

The amount of salt-soluble protein extracted from the meat was measured by homogenizing 15g salted meat with 10 parts 3.18% NaCl solution (w/w) 1 min (final salt concentration = 0.6 M). After standing overnight (5°C), 10 mL of the homogenates were pipetted into glass tubes and centrifuged at 4000 × *g* 10 min. Salt-soluble protein was determined by the Biuret method (standardized against BSA) according to Torten and Whitaker (1964) and reported in mg soluble protein/mg protein.

Statistical analysis

Data were analyzed using analysis of variance (SAS) at the University of Kentucky Agricultural Data Center. Main effects of animal (replication), electrical stimulation and salting time and their interactions were determined and reported where significant ($P<0.05$). Interactions were pooled with the error term when found nonsignificant ($P>0.05$). Pair-wise mean comparisons were made using the protected LSD test (Cochran and Cox, 1957). The effect of salting time was partitioned into linear and curvilinear comparisons using orthogonal polynomials. Simple correlation coefficients were generated to determine if strong associations existed between apparent viscosity, textural and thermal stability traits due to main effects.

RESULTS & DISCUSSION

pH of salted meat

The pH of the salted minces taken immediately after salting (ESI and NSI) decreased ($P<0.05$, linear, quadratic and cubic response) as salting was delayed from 1 to 24 hr postmortem (Fig. 1). The pH values of ES salted meat were lower ($P<0.05$) at each salting time than comparable NS values indicating electrical stimulation accelerated decline in pH. Similar responses have been reported by Fabiansson and Reutersward (1985). Salting inhibited glycolysis in prerigor muscles and stopped accumulation of lactic acid resulting in meat with higher pH (Newbold and Scopes, 1967; Ockerman, 1975). Salting essentially held the pH at the level found at the time of salting in both ES and NS muscles (Choi et al., 1984; Seman, 1988). Using pH 5.9 as a guide to onset of rigor in normal beef muscle (Honikel et al., 1983), we assumed NS muscles salted at 1 and 4 hr were sampled prerigor while ES muscles, salted at 1 hr, were entering rigor. When pH was measured in the same salted minced samples after 48 hr (4°C), similar trends were observed except the range in pH was less; i.e., the 1 hr values for NSI samples were lower while the 24 hr values for both ES48 and NS48 were higher than ESI and NSI. Salting prerigor meat may hold the pH at the time of salting, but it does not appear to do so in absolute terms.

Histological examination

Photomicrographs from uncooked batters produced from meat salted after 1, 4, 8, 12 and 24 hr postmortem from NS carcasses are presented in Fig. 2 A-E; photomicrographs of batters from ES carcasses prepared under the same conditions are shown in Fig. 3 A-F. When the batters were chopped, the lard in the NS samples dispersed rather uniformly throughout the protein matrix regardless of postmortem salting time with no production of clear fat channels, even in the samples prepared after 24 hr postmortem (Fig. 2E). The fat was immobilized as discrete particles (about 10 μm in diameter) surrounded by a thin matrix of muscle protein (Fig. 2 A-D). We therefore, expected little rendering of the lard upon heating to 70°C since the stability of a protein-fat matrix is primarily determined by the fat dispersal pattern developed during comminution (Lee et al., 1981).

Batters prepared from electrically stimulated meat salted 1 hr postmortem (Fig. 3A) had similar fat dispersion patterns containing fat of similar size to that in the NS samples (Fig.

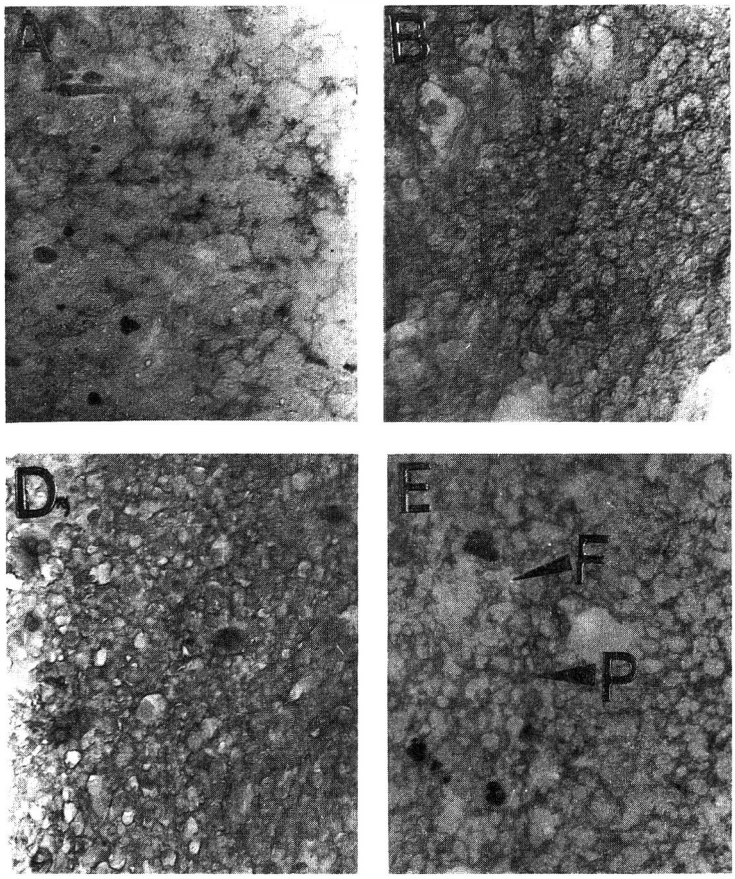

Fig. 2—Photomicrographs of uncooked meat batters prepared from nonstimulated beef minced and salted at different times postmortem. (A) 1 hr, (B) 4 hr, (C) 8 hr, (D) 12 hr, and (E) 24 hr. Magnification: 163x; F = fat P = protein.

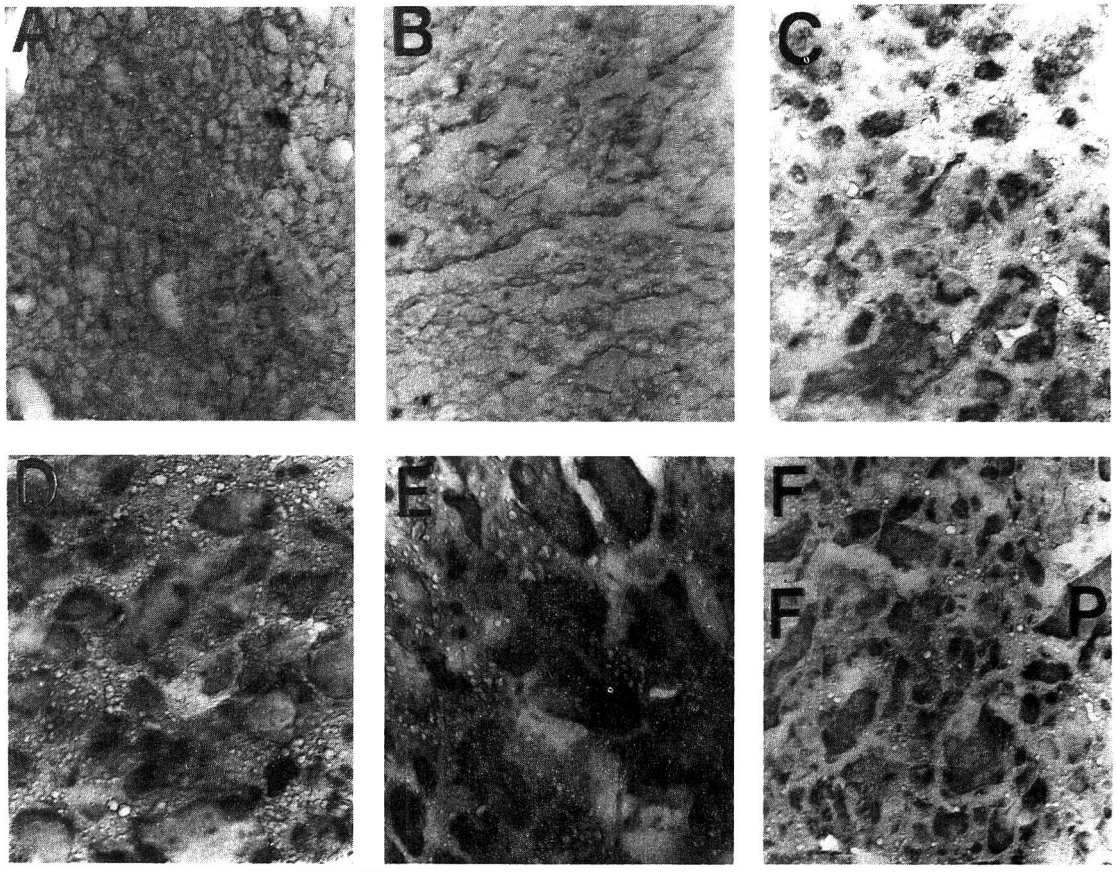

Fig. 3—Photomicrographs of uncooked meat batters prepared from electrically stimulated beef minced and salted at different times postmortem. (A) 1 hr, (B) 4 hr, (C) 4 hr, (D) 8 hr, (E) 12 hr, (F) 24 hr. Magnification: 163x; F = fat P = protein.

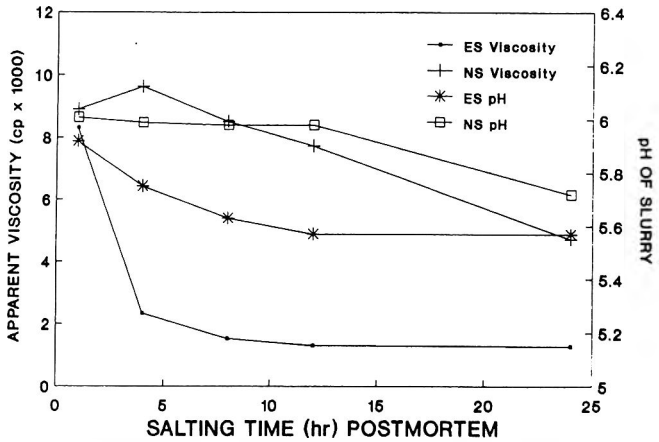

Fig. 4—LSMeans of apparent viscosity and pH of salted meat slurries (containing 200% added water) obtained from electrically stimulated (ES) or nonstimulated (NS) carcasses minced and salted at various times postmortem.

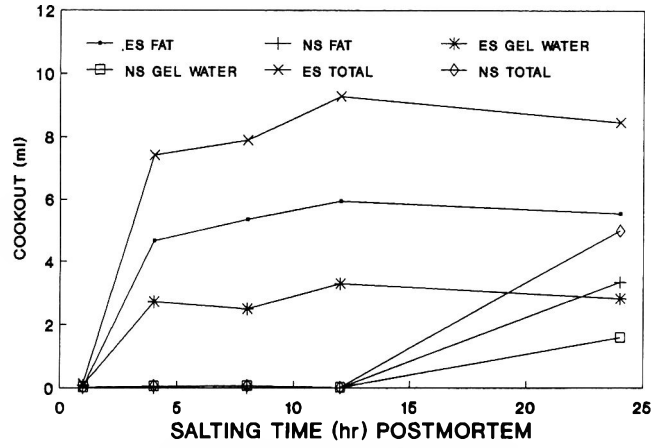

Fig. 5—LSMeans of thermal stability values (ML fat, gel-water and total cookout) of beef batters heated at 70°C for 30 min from electrically stimulated (ES) and nonstimulated (NS) carcasses minced and salted at various times postmortem.

2 A-D). However, delaying salting in the ES meat resulted in lard coalescense leading to formation of fat channels (Fig. 3 B-F). Two types fat dispersion patterns were observed: Fig. 3B illustrates a channeling of fat whereas Fig. 3C-F shows islands of muscle fragments in a sea of fat. The fat dispersal patterns differed drastically from those observed from the NS samples and indicated that unless fat was dispersed in a discrete form during comminution, it would render out upon cooking (Lee et al., 1981). Electrical stimulation exerted a deleterious influence on the ability of hot processed meat to bind fat which can be easily seen by observing the fat dispersal patterns of the raw batter.

Apparent viscosity

The apparent viscosity of meat salted at 1, 4, 8, 12, and 24 hr postmortem is presented in Fig. 4. Samples from ES sides had initial apparent viscosity values of 8,320 cp which rapidly decreased by 4 hr and remained relatively low and constant. These slurries were likened to thin cake batter and would flow easily when the beakers were tipped. The apparent viscosity of samples taken from NS sides was slightly higher ($P<0.05$) initially than the viscosity from ES samples but increased when salting was delayed 4 hr postmortem then decreased. Slurries from NS meat salted at 1, 4, 8 and 12 h did not flow as easily as those from ES sides. Hamm (1986) indicated the viscosity of the continuous matrix played a decisive role in the thermal stability of meat batters and could be affected by pH, addition of salts, rigor state, etc.

The pH values of the slurries used in the determination of apparent viscosity are also presented in Fig. 4. Trends in apparent viscosity generally followed the changes in pH, i.e., samples exhibiting higher pH values also exhibited higher apparent viscosity ($r=0.93$, $P<0.001$). These results confirmed those reported by Toth and Hamm (1968) in which addition of NaOH increased both pH and apparent viscosity of meat slurries containing 80% added water. Thomsen and Zeuthen (1988) also observed increases in pH caused by either addition of mechanically deboned meat or NaOH increased the WHC, yield stress and elasticity modulus in frankfurters.

Although the decrease in apparent viscosity with time postmortem seemed pH dependent, changes in apparent viscosity observed in our study did not consistently follow trends in pH. For example, apparent viscosity in NS at slurries 24 hr was higher ($P<0.001$) than the apparent viscosity in ES slurries at 4 hr while the pH did not differ significantly ($P>0.05$). In addition, a slight increase in apparent viscosity was observed in the NS 4 hr samples while the pH remained unchanged ($P<0.05$). Such results cannot be explained simply by changes in pH; they may be due to effects of electrical stimulation and rigor onset.

Hamm (1975) reported an increase in yield value and apparent viscosity in unsalted muscle homogenates prepared 1 to 24 hr postmortem. After 24 hr, however, yield value and apparent viscosity decreased. That is, viscosity increased during rigor onset even though WHC decreased. Hamm (1975) also reported muscle proteins were not solubilized since these were unsalted homogenates. He suggested the decrease in apparent viscosity may be the result of damage to myofibrillar proteins due to proteolytic degradation during aging. A similar peak in viscosity and yield value at 24 hr was found when salted homogenates (3% NaCl, 50% added water) were prepared at various times postmortem; however, salting reduced the yield value and viscosity when compared to unsalted controls (Hamm and Rede, 1972; cited by Hamm, 1975). The peak in apparent viscosity observed in the NS samples in our study when salting was delayed 4 h may be a consequence of rigor onset causing the myofibrillar proteins to swell, forming a more viscous matrix. Such a matrix apparently did not occur in the ES samples or perhaps it was missed. The decrease in apparent viscosity after the peak at 4 hr was probably the result of decreased WHC caused by formation of actomyosin during onset of rigor mortis as well as decreasing pH.

Thermal stability

Thermal stability data are recorded in Fig. 5. The amount of lard released from ES samples increased ($P<0.05$) as salting was delayed from 1 hr to 8 hr postmortem. However, the amount of cookout did not increase in the NS samples until salting was delayed 12 hr postmortem. The released gel-water increased ($P<0.05$) in ES samples as salting time was postponed, but remained immeasurably low in NS samples until 24 hr postmortem. Total cookout paralleled the gel-water and fat data ($r=0.99$, $P<0.001$; $r=0.0.99$, $P<0.001$; respectively). The amount of cookout (fat, gel-water and total) released from the NS 24 hr samples was less ($P<0.001$) than that from the ES 4 hr samples even though the pH of the salted mince used to form the batters was similar (Fig. 1), again indicating thermal stability was not totally dependent on pH. Electrical stimulation could have caused structural change in actomyosin molecules (Oreshkin et al., 1986).

Textural traits

Gel strength. The puncture resistance (gel strength) test of batter samples from the thermal stability analysis is shown in

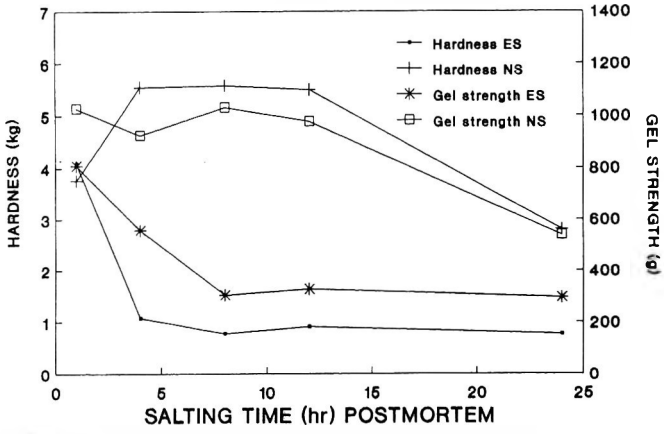

Fig. 6—LSMeans of gel hardness (kg) and strength (g) of beef batters formulated to contain 30% added lard produced from electrically stimulated (ES) or nonstimulated (NS) carcasses minced and salted at various times postmortem.

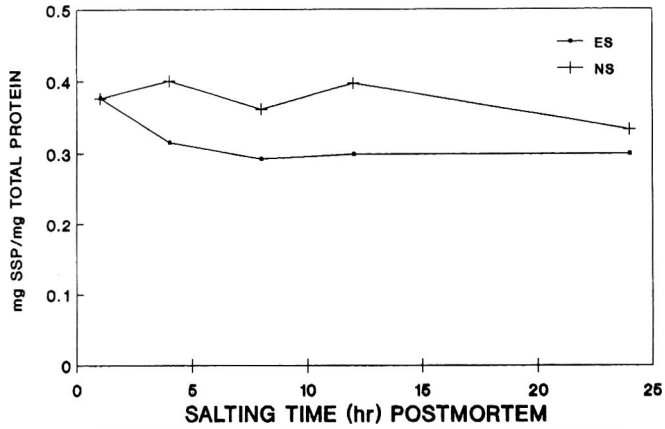

Fig. 7—LSMeans of salt-soluble protein (SSP) content of electrically stimulated (ES) or nonstimulated (NS) carcasses minced and salted at various times postmortem.

Fig. 6. Gel strength in NS samples remained relatively unchanged when salting was delayed 8 hr after which it decreased until 24 hr postmortem. Gel strength of ES samples was initially lower than that of NS samples and decreased after 1 and 4 h as salting time was delayed. Samejima et al. (1986) found myofibrils from fresh ES meat in 0.6M NaCl produced gels with about 40% lower elasticity values than those from myofibrils of NS meat.

Hardness. Hardness values of cooked batter samples are presented in Fig. 6 and indicate the same general trends as found for gel strength ($r = 0.79$, $P < 0.001$). Hardness values of NS samples increased after 1 to 4 h and decreased when salting was delayed after 12 h. Hardness values of samples from ES meat were initially higher than those of NS meat but decreased after 4 h and remained unchanged ($P > 0.05$). Lee et al. (1981) reported compression force decreased with increasing endpoint chopping temperature and decreasing fat hardness because of a weakening of protein matrix which was manifested by formation of fat channels. In our study we observed the increased formation of fat channels and subsequent weakening of gel structure in electrically stimulated meat as early as 4 hr postmortem. However, fat channeling was not observed to the same degree in nonstimulated meat even in 24 hr samples and weakening of the gel structure did not occur until after 12 hr.

Salt-soluble protein

Salting time had little effect on extraction of salt-soluble protein (Fig. 7). The salt-soluble protein content of ES samples was initially the same as for the NS samples but decreased after 4 and 8 hr. Bernthal et al. (1989) also observed prerigor homogenates blended with 2 and 4% NaCl did not change from 0–96 hr postmortem. The amount of salt-soluble protein extracted from meat was reported to decrease with onset of rigor (Acton and Saffle, 1969) and was pH dependent (Saffle and Galbreath, 1964). In this study, however, the slight changes in salt-soluble protein did not seem to have a significant effect on the major differences observed in apparent viscosity and texture traits. They were influenced more by ES and postmortem salting time. Meat/lard batters produced from NS meat salted 1–12 hr postmortem were very stiff and springy and required much scraping to remove from the bowl. Batters produced from ES meat salted at 1 hr were very similar to the NS 1 hr sample but had the texture of cake batter which poured easily from the food processor. The high WHC of the prerigor salted meat gave the continuous phase (protein matrix) suitable structure and viscosity to immobilize the lard and produce a firmer texture when cooked to 70°C. Electrical stimulation negated these effects by speeding postmortem glycolysis (lowering pH).

CONCLUSIONS

THE UTILIZATION of prerigor meat in comminuted meat products has been quite low despite its superior functional properties. These superior functional properties can be maintained in prerigor meat by salting with 1.8% NaCl. If superior functional properties are to be maintained in meat from ES carcasses, the muscle must be salted very quickly (before 1 hr postmortem) otherwise it loses these properties and proceeds into rigor. Raw materials from ES prerigor meat may, therefore, cause variations in final batter texture due to salting time and level added to the meat. The pH of the salted meat can be used as a rough guide to the "functional" quality of the prerigor ES and NS meat but it is likely to change as the meat is held in storage (4°C) and may give misleading results if used as a quality control criterion for rejection. The peaks observed in apparent viscosity and texture data require further study.

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Lactic Fermentation Effects on Preservative Qualities of Dendeng Giling

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ABSTRACT

Model "Dendeng giling", Indonesian non fermented dried beef, was prepared after fermentation with *Lactobacillus plantarum* as starter and compared with control without starter culture. Inoculated sample was fermented at 30°C 16 hr, *Lactobacillus* grew from 10^6 to 10^8, pH went from 5.71 to 5.2. *Micrococcus*, Gram-negative bacteria, *Pseudomonas*, *Staphylococcus*, and *Escherichia coli* were inhibited by 98.3%, 77%, 93%, 96% and 77% after fermentation 16 hr. Inoculated samples were tested for inhibiting VBN and TBA value, dissipating residual nitrite and development of redness. Lower pH caused faster drying at 50°C and reduced water activity. The combination of low pH and water activity made possible improvement of preservative quality.

INTRODUCTION

FOR FOOD PRESERVATION, low temperature to dissipate microbial growth, and high temperature to kill microbes are extensively used by industrialized countries. However, these methods require much energy as well as elaborate equipment and packaging. In tropical countries, therefore, alternative methods for preservation of food products have been based on water activity and pH.

"Dendeng giling," a traditional dried meat product from Indonesia, is prepared by sun drying a mixture of minced beef, salt, garlic, coriander and sodium nitrite (Buckle, 1988). This is a dried product like "Biltong" in South Africa, "Beef Jerky" in North America, "Carne de sol" in South America, "Charqui" in Brazil, "Lup cheong," "Isusou gan," "Nyoursou gan," "Sou song" in China and "Pemmican" in North America (Leistner, 1987). Generally dried meat products undergo many reactions that can cause deterioration including lipid oxidation and off colors during storage (Obanu, 1988). They may contain many additives and residues, which pose legal problems with respect to approval of new additives.

Lactic acid bacteria (LAB) are known for their ability to repress growth of undesirable bacteria by acid production, hydrogen peroxide formation, antibiotics and bacteriocin secretion. They also help dissipate rancidity, residual nitrite and off coloration (Bacus, 1984).

To improve acceptability of "Dendeng giling" on a laboratory scale, it was prepared by inoculating a starter culture, *Lactobacillus plantarum*, and dried in a dehydrator. This specific strain was used because of commercial availability. It has optimal growth at 30–35°C (ambient temperature in many tropical countries) and does not grow at 45°C (sun drying temperature). This strain is salt tolerant, has sucrose fermentative ability and is not subject to extreme acid flavor (Bacus, 1984) when the "Dendeng giling" is high in salt and sucrose.

Our objective was to determine the inhibitory effect of *Lactobacillus plantarum* on some spoilage and pathogenic microorganisms, and to study its role in depressing VBN and TBA values, repressing residual nitrite and color development in "Dendeng giling."

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MATERIALS & METHODS

LACTOBACILLUS PLANTARUM IAM 12316 was incubated in Yeast extract Peptone Glucose (YPG) broth wich contained yeast extract, 5g ; peptone, 10g ; glucose, 10g ; Tween 80, 1g ; L-cysteine, 0.1g in distilled water 1000 mL, pH 6.8. The strain was incubated at 30°C for 18 hr (Nakae et al., 1987) and 10 mL culture was inoculated to 1 kg meat (1%).

Fresh beef was purchased from a local market on the day of preparation. Spices were used as powder (House Co. Ltd.).

Preparation of "Dendeng giling"

Meat was cut and minced in 4 mm diameter with meat grinder MK-G3S (National Co. Ltd.), after removal of the fat. Minced meat was mixed with ingredients containing sugar 10%, salt 5%, coriander 4%, garlic 0.7%, onion 5%, sodium nitrite 0.02% and sodium ascorbate 0.055%. A portion was inoculated with *Lactobacillus plantarum* and the other part was not inoculated, serving as a control sample.

The mixtures of meat were packaged into sterile polyethylene bags (100 g/bag) and molded to plates 2 mm thick, incubated at 30°C for 0, 8, 16, and 24 hr. After fermentation 16 hr, the samples were desiccated by heating at 50°C for 0, 3, 6, 9, and 12 hr to produce model "Dendeng giling." Fermented and desiccated samples for the designated times were used for microbial examinations, chemical analyses and color measurements.

Pretreatment of samples

Dried samples were ground to powder with a homogenizer (AM-8 Nissei Co. Ltd.) 5 min at 15000 rpm. Ten grams were homogenized with 90 mL sterile distilled water with Physcotron (Niti-on Co. Ltd.) 25 sec at 10000 rpm. Ten mL of homogenate was pipetted aseptically for microbial examination, 20 mL for volatile Basal Nitrogen (VBN) analyses, and 70 mL for measuring pH (Acton and Keller, 1974) and Thiobarbituric Acid (TBA).

Microbial examination

One mL of homogenate samples was aseptically diluted stepwise through a series of tubes containing 9 mL sterile buffer saline. Appropriate diluents of each tube sample were placed on the following media in duplicate; MRS agar pH 5.4 (Oxoid Ltd.) for *Lactobacillus* (Kato et al., 1985), Plate Count Agar (PC Difco Co. Ltd.) supplemented with 10% NaCl for *Micrococci* (Steele and Stiles, 1981), CVT agar (Eiken Ltd.) for Gram-negative bacteria (Reddy et al., 1970) Vogel Johnson agar for *Staphylococci*; Bacto of Basal medium which contained: of Basal Medium, 9.4g; yeast extract, 2.0g; Tween 80, 1.0g; agar, 13g in 1000 mL distilled water, pH 7.0. This medium supplemented with maltase (10g in 100 mL distilled water); Nalidixic acid, 7.5 mg and Novobiocin 30.0 mg in 10 mL distilled water, for *Pseudomonas* (Becton Dickinson, 1983) and Desoxycholate agar (Nissui Co. Ltd.) for *E. coli* (Becton Dickinson, 1983).

MRS and PCA duplicate pour plates were incubated at 30°C for 48 hr, Vogel Johnson duplicate surface plates were incubated at 35°C for 48 hr. CVT surface plates were incubated at 30°C for 48 hr, (all black colonies counted and calculated). Of Basal Medium surface plates were incubated at 25°C for 48 hr, and all white colonies counted (Becton Dickinson, 1983). For *E. coli*, duplicate pour plate were made of each dilution using 1.0 mL in Desoxycholate agar. After the pour plates solidified, they were covered with additional 5 or more mL of Desoxycholate agar, incubated at 30°C for 24 hr, and the red colonies counted (Becton and Dickinson, 1983).

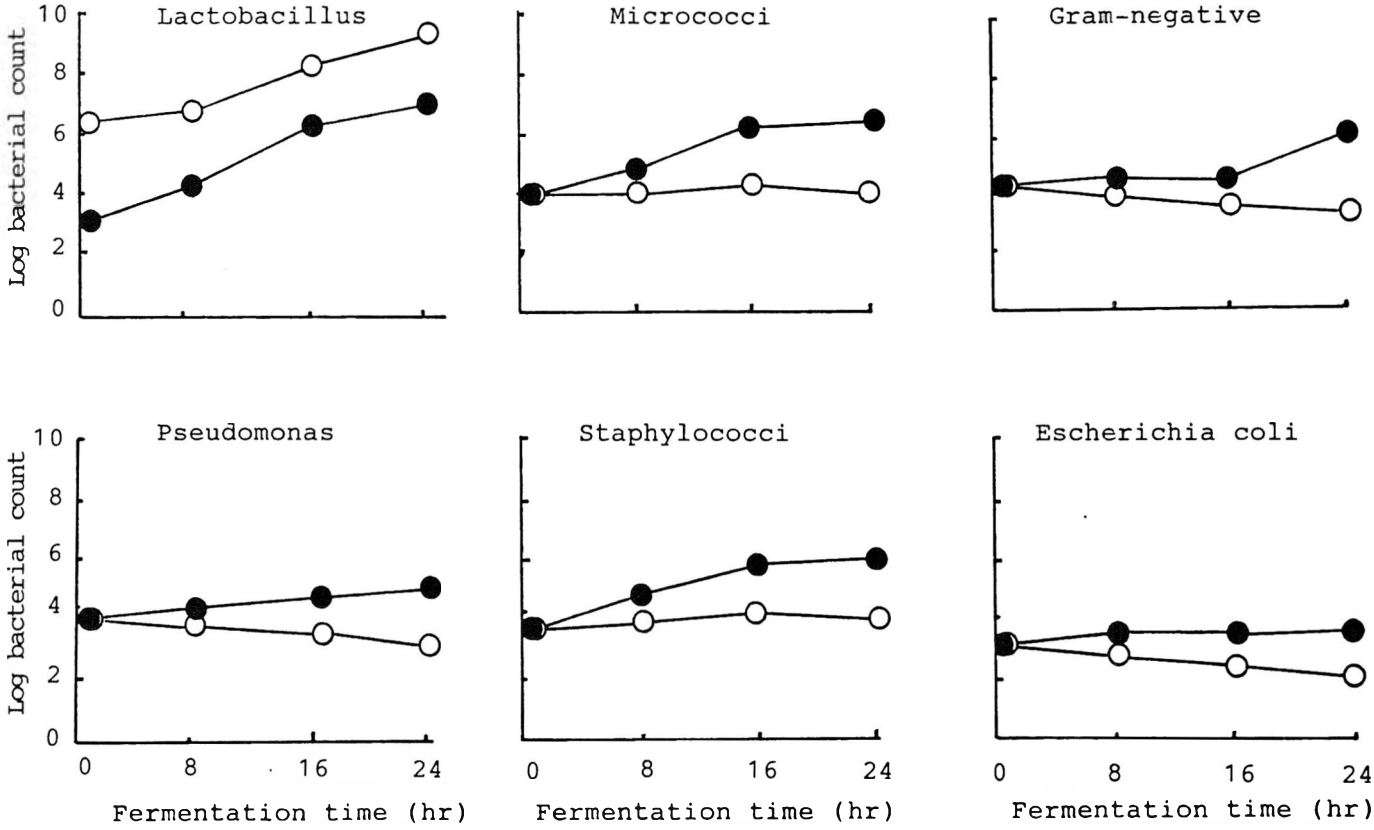

Fig. 1—Growth of bacteria on meat inoculated (○) and not inoculated (●) with Lactobacillus plantarum *during fermentation at 30°C.*

Chemical analysis

Values of pH were measured with pH meter (Horiba F 8). TBA values were determined by a modified Ando method (Ando and Yamauchi, 1968), and expressed as mg malonaldehyde/kg meat. VBN values were measured by Conwey's method (Kousaka, 1983), and residual nitrite as NO_2 measured with Griess reagent (AOAC, 1980). Water content was measured by AOAC method and water activity was estimated with the Conwey's method (Kousaka, 1983).

RESULTS & DISCUSSION

Bacterial growth and pH

Growth of *Lactobacillus plantarum* (MRS count) and its inhibitory effect on spoilage microorganisms during fermentation of "Dendeng giling" is shown in Fig. 1. The growth of this strain increased from 10^6 to 10^8 in 16 hr of fermentation, while the pH decreased from 5.7 to 5.1. On the other hand, the noninoculated sample showed a pH decreased of 0.1 unit (only from 5.7 to 5.6), as shown in Fig. 2.

Inhibition of growth of spoilage and pathogenic microorganisms (i.e. *Micrococci*, Gram-negative bacteria, *Pseudomonas, Staphylococci* and *E. coli*) of inoculated sample compared with noninoculated sample for 16 hr of fermentation were about 98.3, 77.1, 93, 96, and 77%, respectively. This repression became greater with increasing fermentation time. Some researchers have reported lactic cultures had pronounced inhibitory effect on Gram-negative bacteria, which may be associated with synthesis of antibiotic-like inhibitory substances (Reddy et al., 1970). This condition also repressed growth of *Staphylococcus* (Gilliland and Speck, 1977). The dissipating of *Pseudomonas* was possibly caused by hydrogen peroxide, produced and accumulated by *Lactobacillus plantarum* at lower pH (Price and Lee, 1970) and the acid environment of the fermented meat product, played a significant role in stressing and injuring *Escherichia coli* (Frank and Marth, 1977).

Thus, the antibacterial action of *Lactobacillus plantarum* was probably due to a combination of factors including the LAB produced, acid environment, antibiotic-like inhibitory substances such as "Plotulin" and bacteriocin such as "Plantarin A" (Harris et al., 1989) and the hydrogen peroxide activated.

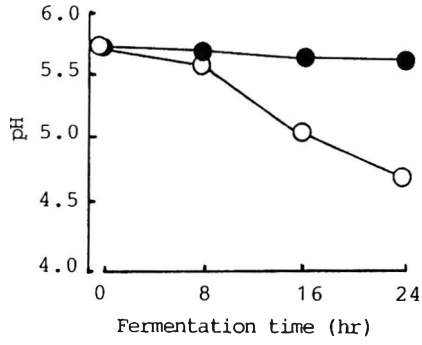

Fig. . 2—Decrease in pH of meat inoculated (○) and not inoculated (●) with Lactobacillus plantarum *during fermentation at 30°C.*

TBA value

TBA value of noninoculated samples increased during fermentation at 30°C for 0, 8, 16 and 24 hr of fermentation. These values went from 0.21, to 0.30, but, the inoculated sample was almost constant during fermentation as shown in Fig. 3. This agreed with Reddy (1975) who reported TBA values of noninoculated samples were higher than those of inoculated samples. Rancidity is caused by the activity of lipolytic microorganisms. Freeman (1960) reported the lipolytic microorganisms were Pseudomonads and Gram-negative. The 5.0% salt and 200 ppm nitrite in our product could adversely affect growth of *Pseudomonas* and other Gram-negative bacteria, and

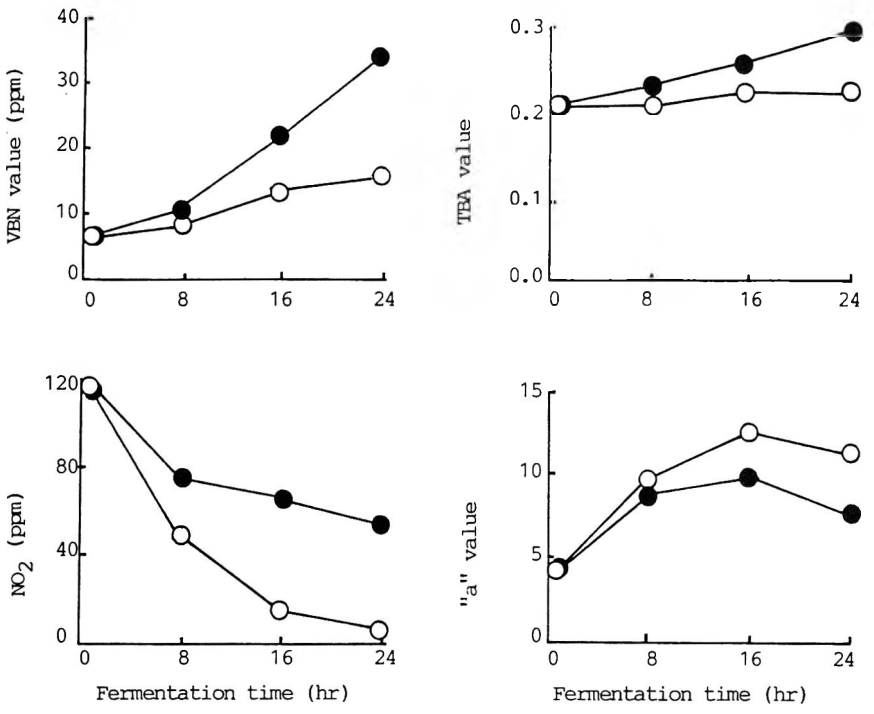

Fig. 3—VBN, TBA, Residual nitrite and "a" values of meat inoculated (○) and not inoculated (●) with Lactobacillus plantarum *during fermentation at 30°C.*

also fermentation by the LAB could inhibit growth of *Pseudomonas* and Gram-negative bacteria.

Another reason, may be the decreasing pH during fermentation increased decomposition of nitrite in inoculated samples faster than in uninoculated samples. However, nitrite tends to oxidize lipids (Jensen, 1954), so lowering residual nitrite in inoculated sample would support the lowering of TBA. In our experiment we suggested roles in the dissipation of TBA value might be played by inhibition of *Pseudomonas* and Gram-negative bacteria and lowering of residual nitrite.

VBN value

Figure 3 showed the VBN value of samples inoculated with LAB during fermentation was lower than in noninoculated sample. Some bacteria, such as *Pseudomonas* secrete proteolytic enzymes which play a role in degradation of protein. This results in meat putrefecation and occurrence of volatile nitrogen. Depression of growth of *Pseudomonas* by LAB fermentation was discussed above, This result suggests that inhibition of *Pseudomonas* by LAB could have repressed the VBN value.

Residual nitrite

Residual nitrite added to the meat decreased immediately about 40% and continued to decrease during fermentation. Residual nitrite decreased more in inoculated samples than in noninoculated samples (Fig. 3). When nitrite was added to meat for curing, less than 63% could be found chemically immediately after addition, and the nitrite in cured meat was found as nitrite, nitrate, nitrothiol, denatured nitroso myoglobin and gaseous nitrogen compounds (Miwa et al., 1976). Tinbergen and Krol (1979) reported the nitrite was strongly reduced by formation of nitrosomyoglobin when the product had been previously heated anaerobically; and the activity was pH dependent, exerting a greater reduction at lower pH. Tanaka et al. (1980) showed the LAB introduced into bacon processing caused decrease in meat pH and supported a reduction in residual nitrite level while maintaining safety of the product. Greenwood (1979) reported low pH and high temperature destroyed the nitrite. He suggested that loss of nitrite was through the alipatic diazoreation (nitrite reacts with an aliphatic amine, in acid medium). Fermentation of sugars of "Dendeng giling" by LAB would be effective to lower pH during processing. This lower pH would repress residual nitrite.

Color

The redness of meat develops during fermentation as shown in Fig. 3. Both inoculated and uninoculated samples had a brown color after mixing with ingredients including nitrite, and demonstrated development of redness after 0 hr fermentation. This agreed with Izumimoto et al. (1982) that in anaerobic condition, met-heme pigment decreased with increasing "a" value. This indicated that metmyoglobin was formed after mixing the ingredients. In anaerobic environment under reducing condition in presence of nitric oxide metmyoglobin was converted to nitrosomyoglobin with time.

In our experiment, the samples inoculated with Lactobacillus plantarum had higher "a" value than the uninoculated samples. Fox and Thomson (1977) reported the overall reaction rate in production of nitric oxide increased with decreasing pH from about 5.5 to 4.5. In acid environment, undissociated nitric acid increased as pH decreased (Freeman, 1960). That means the anaerobic condition and lower pH during fermentation resulted in greater nitrisomyoglobin, which supports the increasing "a" value, resulting in increased redness.

During drying

After fermentation 16 hr, the sample was dried at 50°C for 0, 3, 6, 9 and 12 hr. The fermentation period was limited to 16 hr since taste was too sour under prolonged incubation. Therefore "Dendeng giling" was not a highly sour-tasting product. The drying process decreased water content and water activity. The decrease in the inoculated sample was more rapid than in the noninoculated sample as shown in Fig. 4. This might be caused by decreasing water-holding capacity due to decreased pH resulting from lactic fermentation. Acton and Keller (1974) suggested the minimum water holding capacity was observed around pH 5.0 - 5.2, which almost corresponds to the isoelectric point of the actomyocin. Izumimoto and Kawase (1987) also showed the maximal drip loss in beef muscle was at pH 5.0. Our experiments indicated the decline in pH to 5.1 would be followed by a decrease in water-holding capacity and/or increase in drip loss of beef muscle. This con-

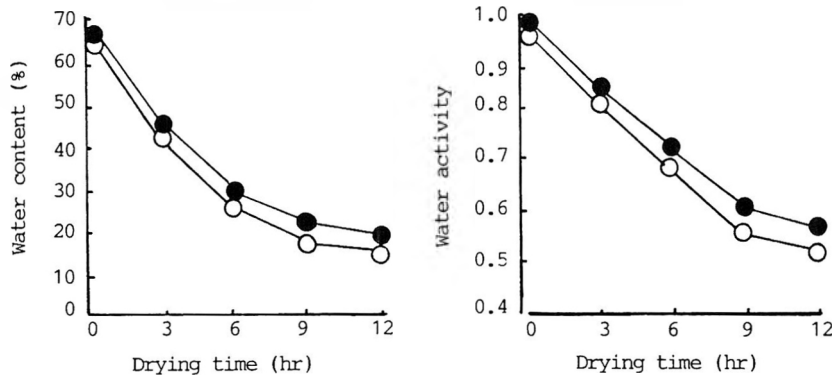

Fig. 4—Water content and water activity of meat inoculated (○) and not inoculated (●) with Lactobacillus plantarum *during drying at 50°C.*

dition promotes a faster drying rate. Thus, the water content and water activity of inoculated samples decreased more than those of noninoculated sample.

Decreasing microbial counts of "Dendeng giling" during drying is shown in Fig. 5. Both the inoculated samples and noninoculated samples decreased in microbial counts during drying. However, the rate of decrease in the inoculated sample was greater than in the noninoculated sample except for *Lactobacillus*. These data agreed with Leistner (1987) who studied Turkish style dried meat (Pastirna). He reported the microbial safety of Turkish Pastirna was superior, because in traditional "Pastirna" the water activity and lower pH were effective, and the competitive LAB contributed to inhibition of microorganisms.

The *staphylococci* and *micrococci* were slower in decreasing of their population. Taylor (1987) reported during processing of "Biltong," salt tolerant nonpathogenic *Staphylococci* and *Micrococci* were dominant microflora of the finished product. This fermentation indicated the *Staphylococci* and *Micrococci* were more resistant than Gram-negative, *Pseudomonas* and *E. coli*. They increased during fermentation, while the other microorganisms decreased.

During drying TBA values and VBN values were in the same proportion as in the fermented products. The values of inoculated samples were lower than those of noninoculated samples; however, the corrected values by water content did not increased "a" value decreased during early drying but increased after 6, 9 and 12 hr as shown in Fig. 6.

Water activity influences activity of the enzyme lipase (David, 1987). At higher water activities, the activity of lipase increased sharply, and was relatively stable at water activity of 0.35–0.80. Enzyme activity was minimal or absent at water activity ≤0.35. This experiment indicated the lower water activity (0.70) repressed lipolytic bacteria and subsequent decrease in lipase enzyme thereby inhibiting rancidity.

Residual nitrite decreased from 16 ppm to 2.6 ppm for inoculated meat and from 66 ppm to 13.6 ppm for noninoculated meat. Decreased nitrite during drying suggested that it was due to decomposition of added nitrite to nitric oxide in the early drying stage. This was followed by development of "a" value,

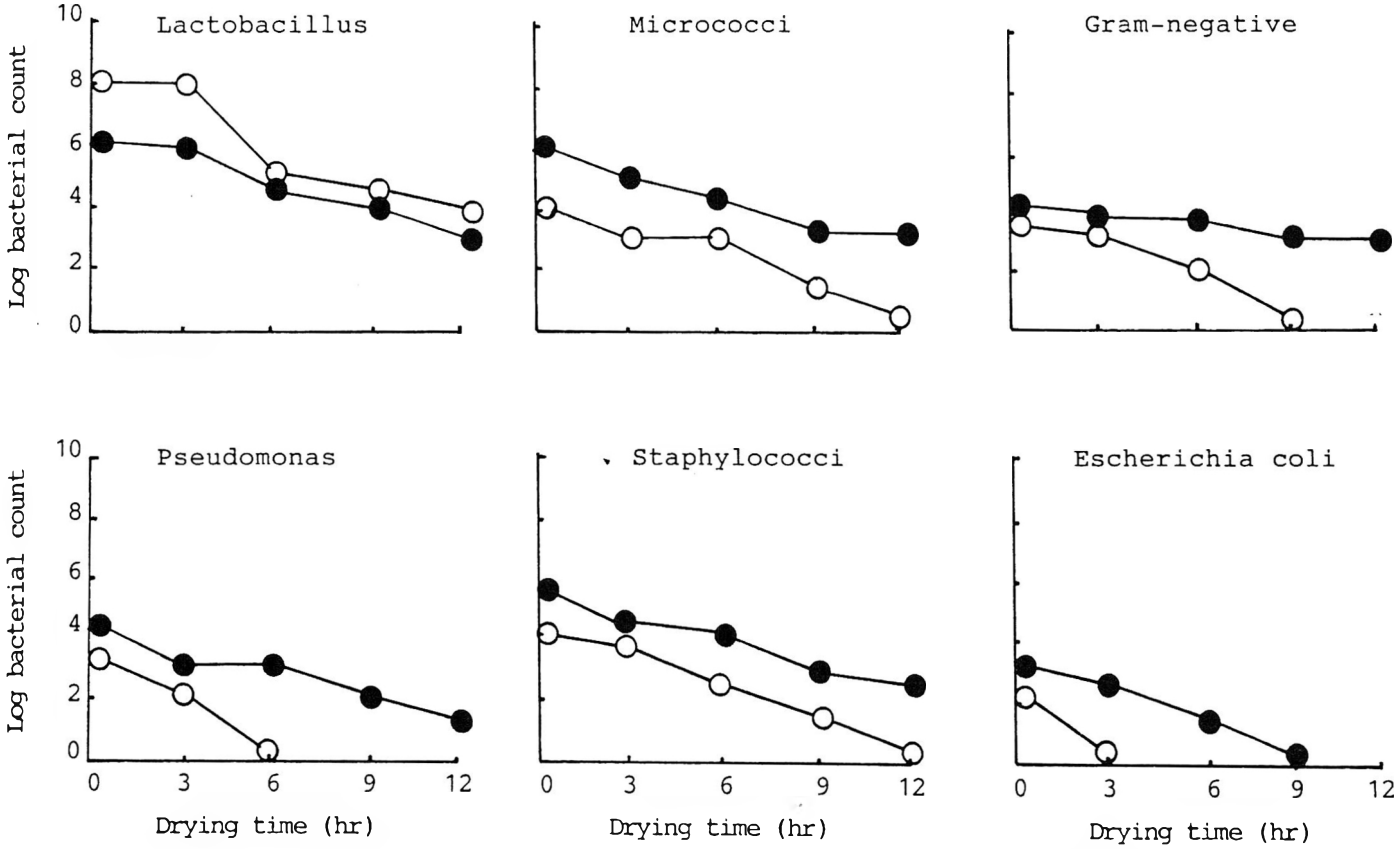

Fig. 5—Decrease of bacterial counts in meat inoculated (○) and not inoculated (●) with Lactobacillus plantarum *during drying at 50°C.*

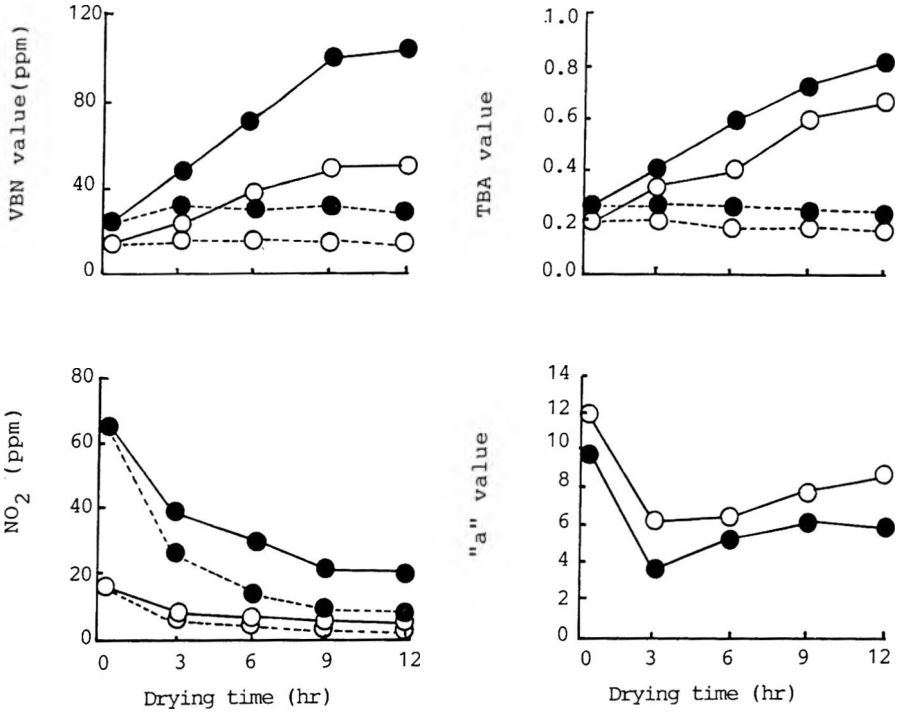

Fig. 6—VBN, TBA, residual nitrite, and "a" values of meat inoculated (○) and not inoculated (●) with Lactobacillus plantarum *during drying at 50°C (-----) corrected with water content.*

as reported by Fujimaki et al. (1975). At start of drying the redness decreased sharply, caused by heme pigment concentration.

The acid condition in the fermented product, produced by LAB, was shown to contribute greatly to destruction of undesirable microorganisms during fermentation, and also to the repression of TBA value, VBN value, dissipation of residual nitrite and increase of color. This means use of LAB would certainly play a role in the total safety system. In combination with reduced water activity, it could provide an effective and economic method to improve preserved quality. Also the application of LAB may be considered in commercial scale production of "Dendeng giling" and other dried meat products.

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Myoglobin Analysis for Determination of Beef, Pork, Horse, Sheep, and Kangaroo Meat in Blended Cooked Products

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ABSTRACT

This method allows the concurrent detection of several types of animal muscle meat in comminuted, cooked meat products. Meat proteins were dissolved in a solvent containing 8M urea and dithioerythritol and separated by electrofocusing on an ultra-thin polyacrylamide gel, containing urea. The proteins were then transferred to nitrocellulose by electroblotting and the blot was incubated with anti-human myoglobin serum, a linking antibody, peroxidase-antiperoxidase (PAP) immune complex, and enzyme-substrate. Detection of less than 10% pork, horse and sheep meat was possible in a beef-based meat product which had been heated to an internal temperature of 120°C for 5 min. The method is not suitable for detection of chicken and turkey meats.

INTRODUCTION

ALTHOUGH many methods have been published to determine nonidentified animal meat in raw meat products, it is more difficult if the meat product has been heat-processed. Differentiation of animal species in cooked meat products requires detecting constituents whose specific determinants either withstand cooking or can be renatured.

Immunochemical and electrophoretic methods are most frequently used. Hayden (1981) developed antisera to heat-stable antigens of horse, pig, sheep, chicken, and turkey adrenals. Immunodiffusion tests indicated these antisera could be used to identify meat from those species at 5–10% in sausages heated to 71°C internal temperature. Cross-reactions occurred between beef and sheep, and between chicken and turkey. Kang'ethe et al. (1986) and Kang'ethe and Gathuma (1987) prepared antisera against thermostable muscle antigens from wild and domestic animals. These antisera were made specific by adsorption with cross-reacting serum proteins. Species identification in heated meat products was achieved by immunodiffusion tests or ELISA. The method is suitable for screening, but the required antisera are not commercially available; they were primarily meant for use in enforcement of the Endangered Species Act (Kenya).

Berger et al. (1988) described an immuno-assay to detect chicken meat and pork in heat-processed meat products. Polyclonal antisera were produced against thermostable protein fractions. The isolation method was similar to the method used for isolating α_1-acid glycoprotein. They detected 1% chicken meat or 1% pork in sterilized beef 'frankfurters'.

In general, electrofocusing is considered a very appropriate technique to identify meat species. By virtue of its high resolving power it can detect small genotypic differences. This high resolving power makes theoretically possible discrimination below the species level. King (1984) combined electrofocusing and zymogram techniques. The meat product was extracted with concentrated solutions of urea or guanidine hydrochloride. After removal of these substances by dialysis, some enzymes (notably creatine kinase and phosphoglucomutase) could be renatured. After electrofocusing and enzyme staining of the renatured extract, they could differentiate between several meat origins.

Hofmann and Blüchel (1986) published a method to differentiate between animal species of raw muscle meat by differences in myoglobin pattern after electrofocusing. The sensitivity of this method could be increased by using peroxidase staining, because myoglobin has pseudoperoxidase activity (Bauer and Hofmann, 1987). This method could not be used to detect species with a low myoglobin content such as chicken and turkey, or samples which had been severely heat-processed. In the latter case the authors recommended silver staining of the protein bands (Bauer and Hofmann, 1989).

By substituting pseudoperoxidase staining for conventional Coomassie Blue or silver staining the number of stained protein bands is dramatically reduced and the pattern is easier to interpret.

Although Bauer and Hofmann could detect nonlabeled species in a heat-processed meat product, the upper temperature limit was rather low because the prosthetic group with the peroxidase activity (the heme) is not covalently bound to the globin. It is split off during the extraction procedure if chaotropic solvents or solvents with high ionic strengths are used. Such solvents are needed to dissolve proteins from severely heat-processed meat products.

Isoelectric-focusing-gels which have been run under denaturing conditions (e.g., high % urea) show a very complex band pattern, which may preclude correct interpretation of mixtures. Therefore, achievement of a reduction in the number of bands is very important. Our work was carried out to investigate whether antigenic determinants on myoglobin, (relatively heat-stable, as well as evolutionarily conserved), could be renatured to the extent that we could use it as a selective marker for meat species identification. Myoglobin had been used as an antigen by Hayden (1979), to detect sheep, pig and horse meat by immunodiffusion in beef products heated to 70°C. Our work also was to lead to a method which would yield permanent results as evidence in cases of legal prosecution.

MATERIALS & METHODS

Reagents

Meat products. Blended meat products were prepared from beef, pork, sheep and horse meat, obtained from local suppliers. Extraneous fat, sinews and tendons were removed, the lean meat was diced and comminuted and each type was mixed with additives as shown in Table 1. The following mixtures were made: beef-pork, beef-sheep, beef-horse and pork-sheep; each with ratios of 90%–10%, 75%–25%, 50%–50%. These mixtures were heated in 5-g portions in flat plastic bags for different times at different temperatures, i.e., 15 min at 80°C,

Table 1—Meats and additives

	%
Meat	89
Curing salt[a]	2
Potato starch	4
Sodium erythorbate	0.05
Water	5
Sodiumpyrophosphate	0.3

[a] 99.4% NaCl, 0.6% $NaNO_2$

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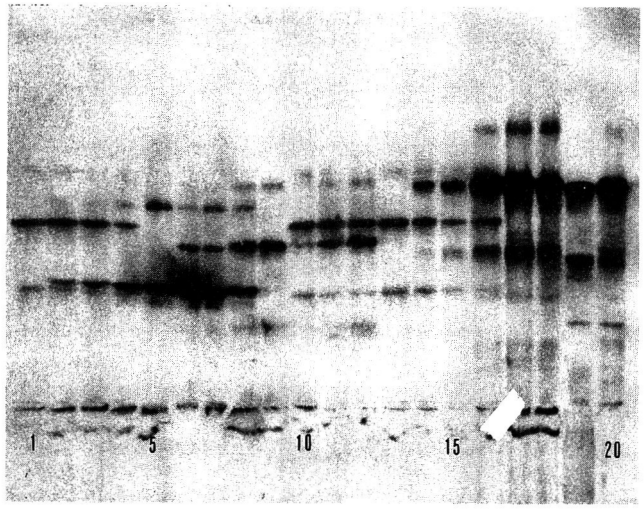

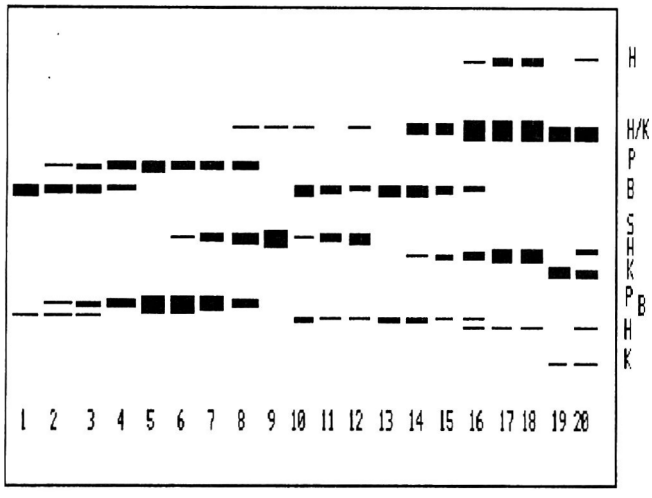

H = Horse B = Beef P = Pork K = Kangaroo S = Sheep

Fig. 1—(from left to right), cathode at top, anode at bottom: 1—beef 100%, 2—beef/pork 90/10, 3—beef/pork 75/25, 4—beef/pork 50/50, 5—pork, 6—pork/sheep 90/10, 7—pork/sheep 75/25, 8—pork/sheep 50/50, 9—sheep, 10—beef/sheep 90/10, 11—beef/sheep 75/25, 12—beef/sheep 50/50, 13—beef, 14—beef/horse 90/10, 15—beef/horse 75/25, 16—beef/horse 50/50, 17—horse, 18—horse, 19—kangaroo, 20—kangaroo/horse 50/50.

15 min at 100°C (water bath) and 5 min (stationary time) at 120°C (autoclave). The mixtures attained these temperatures within a few seconds as the bags were only 1–2 mm thick. Immediately after heating, the bags were cooled with running tap water and stored at −20°C. As only a small amount of kangaroo meat was available, only one sample of meat, heated for 15 min at 100°C was used.

In a separate experiment, meat products (pork, beef) were made with either 2% lactose or 2% glucose and heated for 15 min at 100°C, to determine the effect of non-enzymatic browning on the electrophoretic pattern (isoelectric point). Pig blood plasma and hemoglobin (both dehydrated) were obtained from Harimex-Ligos (Loenen, The Netherlands). All other meat products were bought in local supermarkets.

Antisera. Anti-human myoglobin serum (from rabbit) and anti-rabbit IgG serum (from pig) were obtained from DAKO (Glostrup, Denmark), rabbit-PAP immunocomplex was obtained from either DAKO or American Qualex (La Mirada, CA).

Other reagents. Acrylamide, N, N′-methylenebisacrylamide (Bis), dithioerythritol (DTE), Tween-20 and carrier ampholytes (Servalytes) in the range 3–10 were obtained from Serva (Heidelberg, Germany). Carrier ampholytes in the pH range of 6.5–9 were from Pharmacia (Uppsala, Sweden). Tris (hydroxymethyl)-aminomethane, glycine, urea and 4-chloro-1-naphthol from Merck (Darmstadt, Germany). Nitrocellulose (BA 83) pore diameter 0.2 μm was obtained from Schleicher & Schuell (Dasse., Germany).

Methods

Sample preparation. Samples were prepared by mixing 5–10g meat product in a Waring Blendor 1 min with 50 mL aqueous solvent containing 8M urea and 0.1% DTE as reducing agent. Aliquots of resulting suspensions were centrifuged at 4000×*g* 5 min (Christ laboratory centrifuge). 100 μL of supernatants were transferred to the wells of a 96-well polystyrene microtiter-tray (Greiner, Nürsingen, Germany) which were sealed with strips of adhesive tape. Reference samples, heated meat of one species or blends of several species, were prepared in a similar manner.

Electrofocusing. Electrofocusing was carried out on a LKB Ultrophor 2217 apparatus with a LKB Macrodrive-5 power supply (Pharmacia, Uppsala, Sweden). Polyacrylamide gels (5% acrylamide, 0.15% Bis) 260×125×0.25 mm were cast on the *hydrophobic* side of Gelbond foils (FMC, Rockland, ME). The gels contained 6.5M urea and 3% of a mixture of ampholytes (Servalyt 3–10 and Pharmalyte 6.5–9 in a ratio of 1:5). Anode fluid consisted of 1M H_3PO_4, cathode fluid of 1M NaOH. After prefocusing at power setting 3 W 15 min., 1–2 μL of sample or reference extract was applied to the gel, approx. 1.5 cm from the anode. Focusing was completed in 1.5 hr at power setting 6 W, which resulted in initial voltage of 850 V and 2000–2100 V at the end of the run.

Blotting. The proteins were transferred to nitrocellulose by electroblotting (Towbin et al., 1979). After removal of the electrode and sample strips, a piece of dry filter paper (Schleicher & Schuell 3469) was pressed firmly against the gel and left for about 1 min. By lifting the filter paper carefully, the gel could be peeled from the supporting polyester foil. The blotter/gel was assembled in a tray containing transfer buffer (2.8g tris (hydroxymethyl) aminomethane, 15g glycine and 100 mL methanol/liter).

The filter paper with the gel was first carefully wetted with transfer buffer to prevent inclusion of air bubbles between the gel and the paper. A piece of polyester sieve printing screen (Schleicher & Schuell TG 100) was placed on top of the gel surface, followed by a piece of nitrocellulose (after prior wetting of the screen and the nitrocellulose with transfer buffer). The screen prevented the gel from sticking to the nitrocellulose foil after the blotting procedure.

After placing filter paper and Dacron sponges on both sides, the sandwich was clamped together in a cassette. The assemblage was subsequently inserted into a blotting tank (Bio Rad trans blot cell, Richmond, CA) containing transfer buffer. Blotting was carried out overnight at a voltage of 40 V (Bio Rad model 250/2.5 power supply). After blotting, the sandwich was disassembled and the nitrocellulose foil was washed in blocking buffer (2.56g $Na_2HPO_4 \cdot 2H_2O$, 0.87g $NaH_2PO_4 \cdot 2H_2O$, 18g NaCl and 6 mL Tween 20/2L) for 10 min.

Staining. Selective staining was achieved by a (double) peroxidase-antiperoxidase protocol (Sternberger et al., 1970), as previously described (Janssen et al., 1986, 1987). All antiserum dilutions were made in blocking buffer. Incubations were carried out in small plastic envelopes which were heat-sealed after insertion of the nitrocellulose blot and the addition of incubation fluid. The volume of incubation fluid could thus be kept to 1 mL/20 cm^2 nitrocellulose foil. All washing steps were carried out in petri dishes on a rocking table. The substrate solution was prepared by dissolving 25 mg 4-chloro-1-naphthol in 10 ml ethanol, adding 40 mL Tris/HCl buffer (0.01M, pH 7.6) and 10 μL H_2O_2 (30%). The following sequence of incubations was used: (1) 1:250 dilution of anti-human myoglobin (rabbit) (15 min); (2) Wash with blocking buffer (4 × 1 min); (3) 1:100 dilution of anti-rabbit IgG (pig) (15 min); (4) Wash with blocking buffer (4 × 1 min); (5) 1:250 dilution of rabbit PAP (15 min); (6) Repetition of steps 2 thru 5; (7) Wash with Tris/HCl buffer (0.01M, pH 7.6) (4 × 1 min); (8) Incubation with substrate (H_2O_2 and 4-chloro-1-naphthol).

RESULTS & DISCUSSION

Results

The results are shown in Fig. 1 and 2. Figure 1 and 1a shows several binary mixtures of pork, sheep, horse or beef which had been heated for 5 min at 120°C, as well as kangaroo meat and a mixture of horse and kangaroo meat, heated 15 min at 100°C. The pH gradient enabled detection of all five meat species simultaneously. Experiments with samples heated

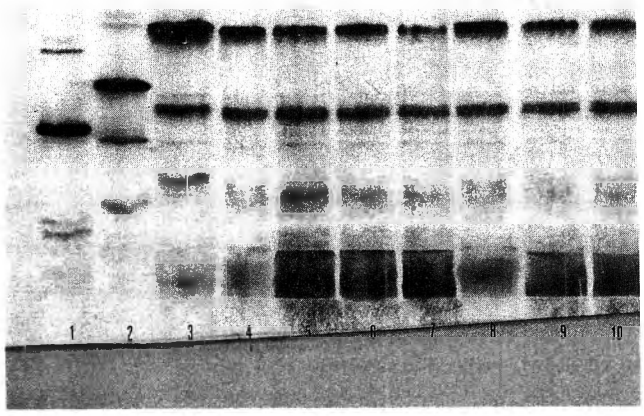

Fig. 2—(from left to right), cathode at top, anode at bottom: 1—pork, 2—beef, 3—horse meat (5' 100°C), 4—horse meat (45' 100°C), 5—meat croquette, 6—meat croquette, 7—meat croquette, 8—horse meat (45' 100°C), 9—6 + 8 (50/50), 10—7 + 8 (50/50),

Table 2—Products analyzed

Meat product	Labeled	Detected
croquette (2)	beef	beef
croquette (3)	meat	horse
smoked sausage (6)	pork	pork
pate	pork	pork
kalabasja	beef/pork	beef/pork/horse
minced meat	veal	–
minced meat	veal	beef
filet cheval	horse	horse
rolled veal	veal	beef
tongue sausage	pork	pork
salted meat	beef	beef
sausage	veal/pork	beef/pork
sausage	horse	horse
frankfurter	beef/pork	pork
frankfurter (4)	beef/pork	beef/pork
frankfurter (2)	pork/chicken	pork
frankfurter (2)	beef/pork/chicken	pork
frankfurter	beef/pork/chicken	–
frankfurter	pork	pork
goulash	beef	–
sausage	pork	pork
liver sausage	beef/pork	pork

15 min at 80°C and at 100°C revealed the same band pattern (results not shown). Heat treatment did not affect electrophoretic pattern of myoglobins which confirmed results of Bauer and Hofmann (1987).

Blood plasma (pig) and hemoglobin powder (pig) did not cross-react with the anti-human myoglobin serum; neither was the band pattern disturbed by products of Maillard browning. Figure 2 displays part of the results obtained with some meat croquettes. It was striking to note that when meat species was not on the label, the croquettes were entirely from horse meat. All croquettes examined, which were labelled as containing beef, consisted exclusively of beef. Several meat products were analyzed (Table 2). In most cases, the species recorded on the label was detected; in some cases the results did not correspond to labeled ingredients or no myoglobin bands could be detected.

Discussion

To detect meat of nonlabeled species by immunoassay, generally requires antisera which show an absolute specificity towards antigens of the species to be detected. Detection systems based on these antisera have the disadvantage that only the meat species being sought are detected.

In our tests the myoglobins of the species all cross-reacted with the anti-human myoglobin serum as shown in Fig. 1. Atassi (1977), in investigating the antigenic structure of myoglobins, observed myoglobins from different species showed different degrees of cross-reactivity with anti-human myoglobin serum. This cross-reactivity was related to phylogenetic difference. We concluded from Fig. 1 that many species show more than one protein band binding to anti-human myoglobin serum. This is in agreement with observations of Hofmann and Blüchel (1986) who assumed existence of myoglobin isomers.

The degree of oxidation of the heme moiety of the myoglobin molecule does not explain the results, as the heme was split off during extraction. An immunochemical cross-reactivity with proteins of unknown identity cannot be excluded. We found due to either (1) a very low myoglobin content, or (2) a more distant phylogenetic relationship between poultry and man, detection of chicken and turkey in beef-based meat products proved impossible.

As meat from other fowl (wild duck, partridge, goose and dove) gave a positive reaction with the anti-human myoglobin serum (preliminary experiment) probably the low content of myoglobin in chicken meat is the responsible factor.

Detection of meat from rabbit also proved impossible (results not shown). This is probably because the anti-human myoglobin serum we used had been produced in rabbits. Hofmann and Blüchel (1986) reported that muscle from different parts of the same animal displayed similar electrophoretic (IEF) myoglobin patterns. Effects of heat processing of the meat in the presence of reducing sugars were not observed.

The quantitative aspects were difficult to validate. The intensity of the myoglobin bands was not only dependent on severity of heating, but also on factors which affect myoglobin content of the animal (e.g., nutrition, maturity). Other published methods have these same limitations.

Though immunochemical methods are generally very specific, specificity as such is not a prerequisite for our method. The required specificity comes from the combined power of separation by isoelectric focusing and immunological detection. An advantage of this method is that there is no need for antisera of absolute specificity.

A second advantage is that a semi-permanent record of results is obtained, which might be very helpful to law enforcement authorities. The most important advantage, compared to ELISA, is that the results do not rely completely on specificity of the antisera used. The proposed method is therefore especially suitable as a confirmatory test on suspect samples.

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Effect of Pressure Cooking and Pressure Rate Change during Cooling in Vacuum on Chicken Breast Quality and Yield

K. P. SELF, G. R. NUTE, D. BURFOOT, and C. B. MONCRIEFF

ABSTRACT

The effect was studied of processing time, weight loss, quality, cooking in steam at a pressure of 1.03 or 0.345 bar and cooling at different pressure reduction rate (0.6 to 0.07 bar/minute) on chicken breasts quality. Heating time was not affected by cooking pressure but cooling time was reduced as pressure reduction rate increased. Weight losses varied from 26.4 to 37.7% and increased with severity of temperature-time treatment. Tenderness was not affected by cooking/cooling but juiciness decreased with increased pressure and reduced cooling rate. Juiciness correlated with weight loss ($r = -0.72$). A 9 member sensory panel indicated harsher temperature-time treatments gave more acceptable meat.

INTRODUCTION

THE MARKET for poultry has grown steadily over the past 10 years and chicken currently commands 78% of the volume sales in the UK (Anon, 1989). Demographic changes and the demand for more convenience foods have led to an increased requirement for pre-cooked products. The cooking/cooling process must produce a safe product of required texture and sensory properties with economic yield.

Oven roasting and deep fat frying are the most common cooking methods for poultry, but steam cooking has been reported. In comparison with water cooking, Yingst et al. (1971) used steam between 0 and 1.03 bar (gauge pressure) to cook chicken parts (breasts, thighs, drumsticks and wings). They found cooking to an internal temperature of about 85°C followed by slow release of steam pressure resulted in losses from unbreaded parts of 15 to 16.5%. Cooking times of 12 minutes at 0.345 bar (5 psi) and 8 minutes at 1.03 bar (15 psi) were reported in the same work. Cooking method using different steam pressures did not affect shear values of cooked meat. They were all within the "tender" range. Klose and Mercuri (1973) investigated sub-atmospheric steam pressure and temperatures from 71 to 100°C to cook poultry parts to internal temperatures of 71 to 85°C. At higher steam temperatures, 12 minutes were required to heat a 134g chicken thigh to 82°C. Weight losses ranged from 13.4 to 24%.

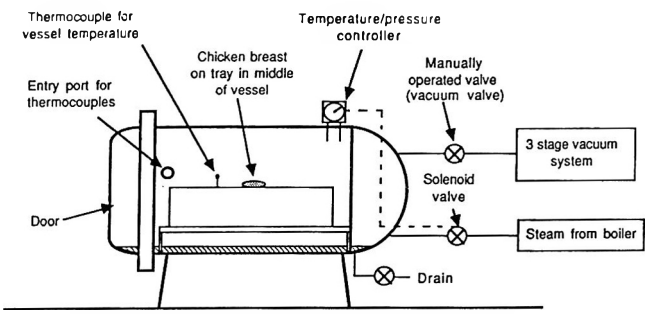

Fig. 1—APV steam pressure cooker/vacuum cooler.

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Methods of cooling meat products include air cooling, water immersion, and cryogenic techniques, but vacuum cooling has been reported for other foodstuffs. Longmore (1970) cooled horticultural products, such as lettuce and mushrooms, from ambient to chilled temperatures. Vacuum chilling of post-slaughter broiler carcasses was reported by Hofmans and Veerkamp (1976), who found the cooling time from 40 to 10°C was about the same as air cooling at a velocity of 2.5 m/s.

Table 1—Allocation of chicken breasts to cooking/cooling treatments (Two breasts from each bird)

Cooking pressure	0.345 bar (5 psi)		1.03 bar (15 psi)	
Cooling rate	Slow	Fast	Slow	Fast
Breast position	Right	Left	Right	Left
Session				
1	Bird 1		Bird 2	
2	Bird 3		Bird 4	
3	Bird 5		Bird 6	
4	Bird 7		Bird 8	
5	Bird 9		Bird 10	
6	Bird 11		Bird 12	
7	Bird 13		Bird 14	

Table 2—Category scales used in the assessment of cold chicken breast. Scores (in parentheses) were allocated after testing

Color of lean		**Chicken flavor intensity**	
Extremely dark	(+7)	Extremely strong	(+7)
Very dark	(+5)	Very strong	(+5)
Moderately dark	(+3)	Moderately strong	(+3)
Slightly dark	(+1)	Slightly strong	(+1)
Slightly pale	(−1)	Slightly weak	(−1)
Moderately pale	(−3)	Moderately weak	(−3)
Very pale	(−5)	Very weak	(−5)
Extremely pale	(−7)	Extremely weak	(−7)
Tenderness		**Foreign flavor intensity**	
Extremely tender	(+7)	Pronounced foreign flavor	(+3)
Very tender	(+5)	Moderate foreign flavor	(+2)
Moderately tender	(+3)	Slight foreign flavor	(+1)
Slightly tender	(+1)	No foreign flavor	(0)
Slightly tough	(−1)		
Moderately tough	(−3)		
Very tough	(−5)	**Flavor liking (Hedonic)**	
Extremely tough	(−7)	Like extremely	(+7)
		Like very much	(+5)
Juiciness		Like moderately	(+3)
Extremely juicy	(+4)	Like slightly	(+1)
Very juicy	(+3)	Dislike slightly	(−1)
Moderately juicy	(+2)	Dislike moderately	(−3)
Slightly juicy	(+1)	Dislike very much	(−5)
Dry	(0)	Dislike extremely	(−7)

Overall acceptability (Hedonic)

Extremely acceptable	(+7)
Very acceptable	(+5)
Moderately acceptable	(+3)
Slightly acceptable	(+1)
Slightly unacceptable	(−1)
Moderately unacceptable	(−3)
Very unacceptable	(−5)
Extremely unacceptable	(−7)

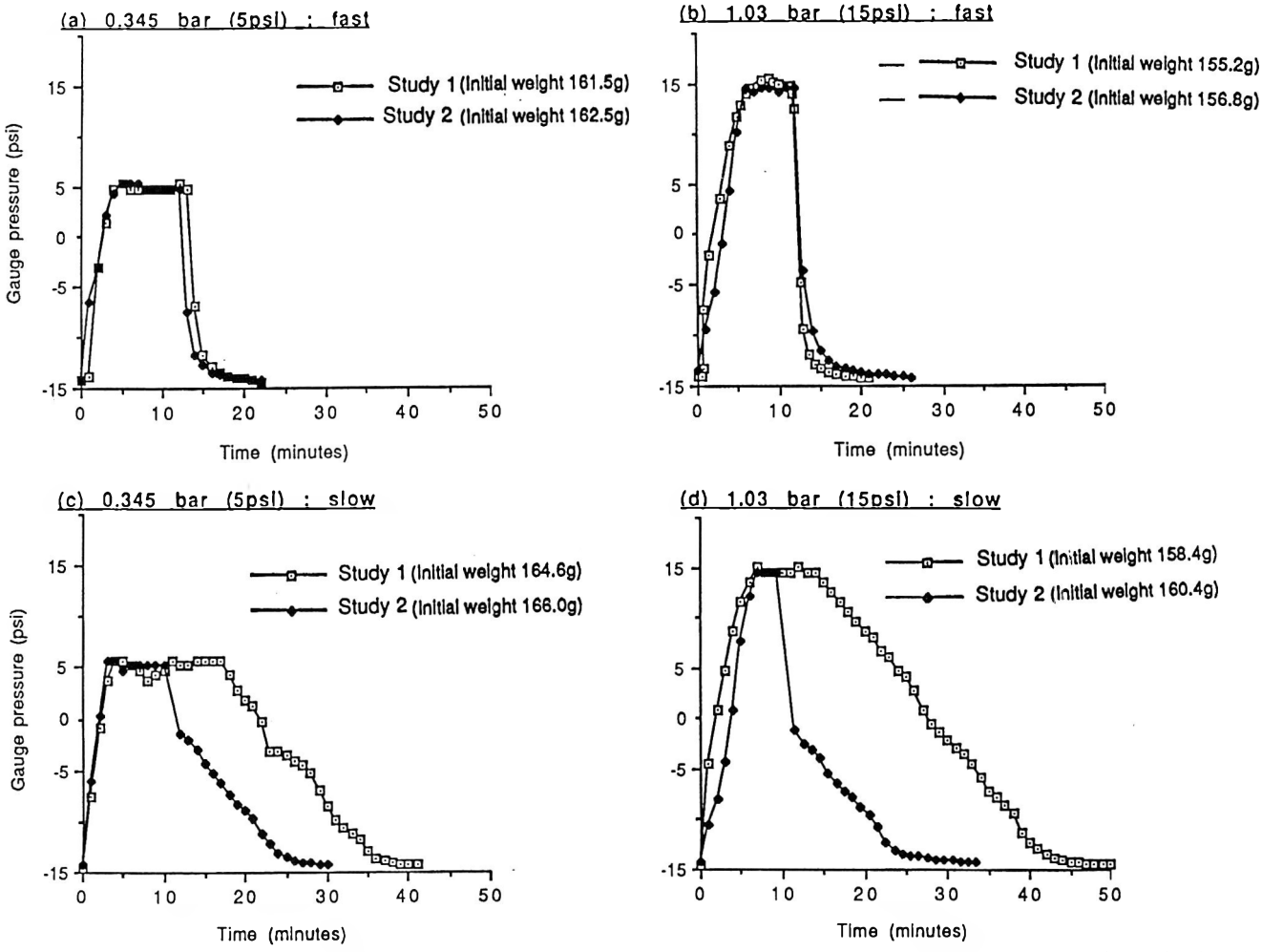

Fig. 2—Typical vessel pressure for cooking/cooling conditions. (Corresponding initial weight of breasts in each experiment is given.)

Table 3—Mean weights, heating times, internal temperatures, cooling times, and weight losses for chicken breasts cooked by steam at 5 psi or 15 psi gauge pressure, and cooled by fast or slow vacuum method (Standard deviation in brackets)

	Initial weight (g)	Heating time to 80°C (Minutes)	Maximum internal temp (°C)	Cooling time, 80 to 30°C (minutes)	Weight loss (%)
Study 1					
Cooking pressure (psi)/Cooling rate					
5/fast	144.2 (18.9)	11.8 (1.4)	83.4 (0.6)	9.9 (1.0)	26.4 (1.9)
5/slow	150.2 (21.7)	13.4 (3.2)	95.3 (2.1) b	24.1 (1.0) b	31.5 (3.0) a,b
15/fast	156.0 (21.6)	12.2 (2.0)	84.0 (2.0)	10.3 (1.2)	30.5 (3.8)
15/slow	160.6 (26.2)	13.0 (3.0)	106.8 (1.4)	38.2 (1.4)	37.7 (2.9)
Study 2					
Cooking pressure (psi)/Cooling rate					
5/fast	176.2 (9.3)	17.9 (1.3)	78.9 (1.3)	11.0 (2.2)	28.5 (2.7)
5/slow	169.0 (13.5)	15.7 (2.6) a	83.1 (1.5) b	16.3 (2.3) b	27.6 (3.0) a
15/fast	169.9 (8.8)	14.7 (1.3)	80.9 (1.5)	13.2 (2.1)	31.4 (3.9)
15/slow	163.0 (7.8)	14.7 (0.7)	85.0 (1.0)	18.5 (2.3)	31.5 (3.0)

[a] Significant difference between 5 psi (0.345 bar) and 15 psi (1.03 bar) at 5% level
[b] Significant difference between fast and slow cooling rate at 5% level

Weight losses during vacuum cooling were high, about 1% for every 7°C reduction. Everington (1977) vacuum cooled offal to 10°C after steam surface pasteurization. This was the only reference found on vacuum cooling of solid meat products from high temperatures after cooking, but the available abstract provided few details.

Initial experiments indicated whole poultry cooked at high pressure and subsequently cooled very rapidly by a vacuum method would show signs of disintegration. The principal aims of our investigation were to determine if steam pressure during cooking and evaporative rate during cooling affected the tenderness, sensory quality and yield of chicken breasts.

MATERIALS & METHOD

Processing vessel

A combined steam cooker/vacuum cooler manufactured by APV Parafreeze, England (Fig. 1) was the processing vessel. The main components were a chamber in which the product was placed, a steam supply at 5.52 bar (80 psi) and a 3-stage vacuum system consisting of a water ring pump, steam ejector and direct condenser. After loading with a chicken breast, the vessel was evacuated 4 minutes and then supplied with steam to obtain the required heating and cooking treatment. Vessel temperature during heating was maintained at a set point by a temperature controller operating a valve from the boiler. The controller could also be used to maintain a set pressure following

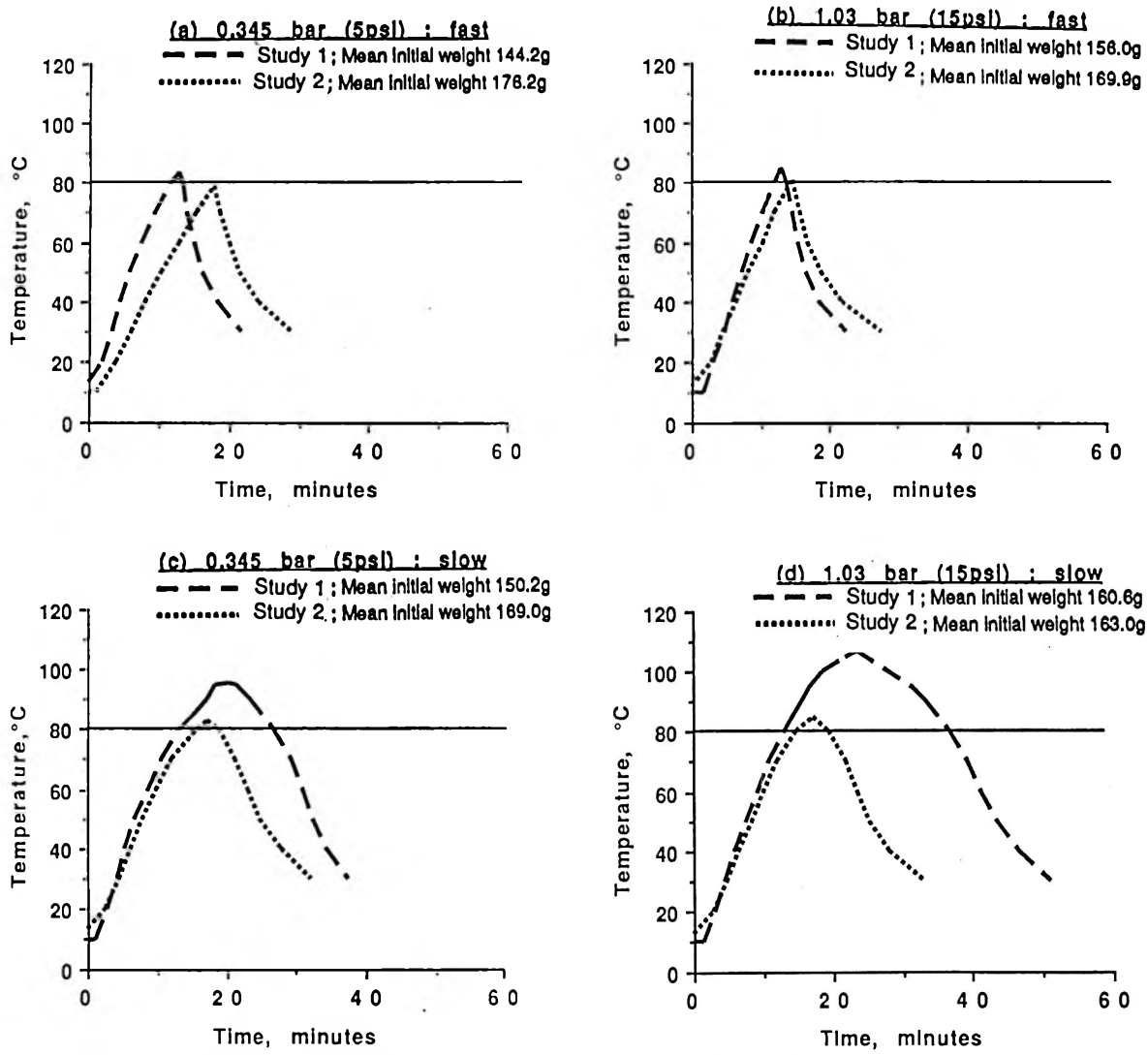

Fig. 3—Minimum temperature recorded in chicken breasts cooked at either 1.03 or 0.345 bar and cooled by either a fast or slow pressure release rate. Mean temperature profiles from seven replicates.

initial evacuation because the subsequent temperature in the vessel would be the saturation temperature at the prevailing pressure. Following the required heating and cooking treatment, cooling was applied using the vacuum system.

Instrumentation

Product temperature was measured by inserting a 7-point thermocouple probe (seven chromel/alumel thermocouples, 0.076 mm diameter, spaced at 8 mm equidistant intervals, along a 3 mm diameter wooden rod) through the thickest part of the breast. Vessel temperature was monitored by a calibrated chromel/alumel thermocouple (0.2 mm diameter) placed 150 mm from the samples. All thermocouples were connected to a data logging system (Magus Measurement Systems Ltd, England). A Bourdon gauge (accuracy ± 20 mbar) was used to measure pressure above atmospheric and a digital vacuum gauge transmitter (Edwards Vacuum Products, England, accuracy ± 2 mbar) was used to measure vacuum pressures.

Chicken breasts

Freshly slaughtered Cobb chickens reared under controlled conditions 40 days were obtained from a local plant and held at 0–5°C for 3 to 5 days. The dressed weight of the birds was 1.6 ± 0.1 kg. A pair of breasts (average weight = 161g) were removed from each bird and held in a chillroom at 0–5°C for another 1 to 6 hours.

Method

Breasts from each bird were allocated to a single heating treatment (0.34 or 1.03 bar) and one breast from each pair was allocated to a fast or slow cooling treatment (Table 1), Prior to each test the vessel was preheated to 50°C and one weighed chicken breast with a temperature measuring probe inserted, placed on a stainless steel tray in the middle of the vessel.

The internal breast temperature and vessel temperature and pressure were recorded throughout the experiment at 30s intervals with temperatures displayed on a monitor at 5s intervals. Once the vessel had reached the desired working pressure the automatic temperature control on the vessel was used to maintain required pressure.

Two studies were carried out. In the first, heating was continued until the internal temperature reached 80°C, this being the minimum measured in the breast at any time during heating (maximum during cooling). The steam supply was stopped and a fast or slow cooling treatment applied. Fast cooling consisted of opening the vacuum valve fully, whereupon the pressure release rate was almost 0.06 bar per minute. Slow cooling consisted of manually regulating the valve between the vacuum plant and the vessel so that the pressure release rate was 0.07 bar/min. At low vacuum in the vessel (<100 mbar) the operating characteristics of the vessel and vacuum plant made it impossible to maintain these pressure release rates, and further cooling was continued with the vacuum valve fully open. With either cooling rate this method resulted in the internal breast temperature after cooking rising above 80°C.

In the second study, carried out after the first was completed, the same procedure was followed except heating was stopped prematurely

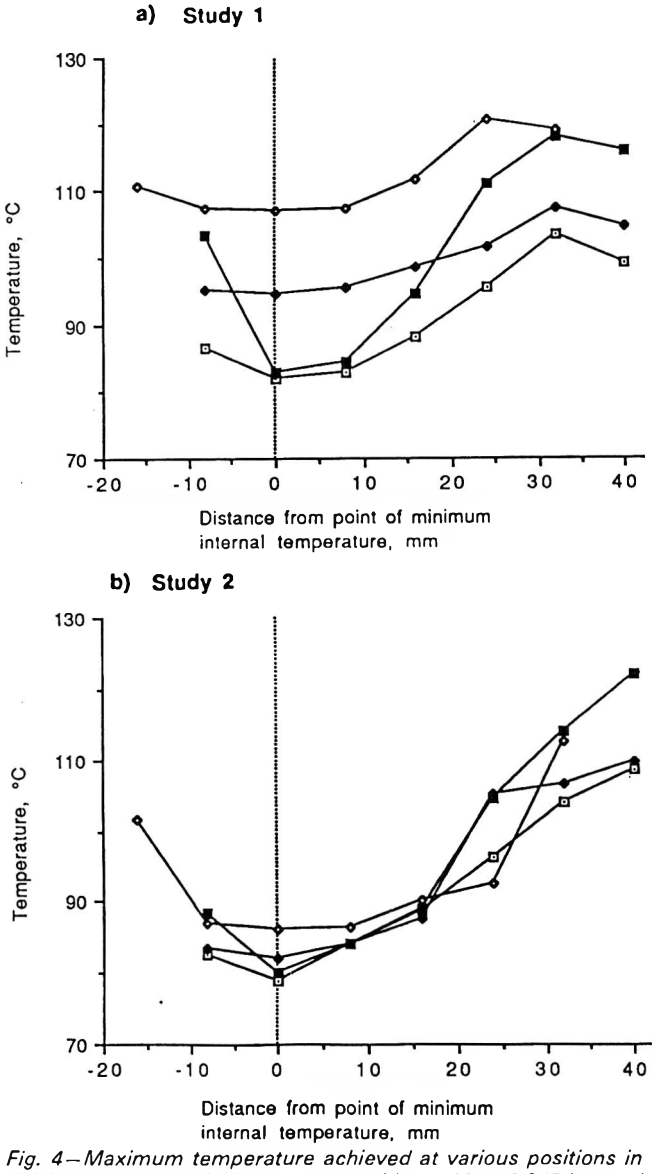

Fig. 4—Maximum temperature achieved at various positions in chicken breasts cooked by steam at either 1.03 or 0.345 bar and cooled by either a fast or slow pressure release rate (□—□ 0.345 bar, fast: ♦—♦ 0.345 slow: ■—■ 1.03 bar, fast: ◇—◇ 1.03 bar, slow)

to try to eliminate the rise in temperature above 80°C. For fast cooling, heating was stopped at an internal breast temperature of 75°C. During slow cooling, we had to (a) reduce the vessel pressure to atmospheric before commencing the slow rate of vacuum release, and (b) stop the steam supply when the breast reached an internal temperature of 58°C. This was sufficient to ensure a final internal temperature of 80 ± 5°C was always achieved.

For both studies, cooling continued until the measured internal breast temperature was 30°C. The vacuum valve was then shut, the vacuum released and the chicken breast removed and weighed. The breast was placed in a polyethylene bag and held 16 to 21 hr in a chillroom at 5 ± 2°C. The chicken breasts from each of the four experimental conditions × seven replicates were submitted for sensory analysis.

Sensory analysis

A panel of nine members (all women) were used to carry out the sensory tests on the chicken breasts. Each had been screened for their basic taste and color acuity and had previous experience on sensory testing of meat and poultry. Prior to service, two training sessions were used to familiarize panelists with the range of samples they would encounter and the category scales used (Table 2). Two hedonic scales were also included to gain some indication of panelists preference for flavor and overall acceptability, although with a small panel the scores only gave an indication and do not reflect potential consumer response.

The same panelists were used for both studies and each of seven replicates. At each session, panelists were presented with four samples of cold chicken breast (5 mm thick slices cut at right angles to the sternum), one from each pressure/cooling combination. Samples were presented in randomized order.

Statistical analyses

Scores were awarded to the category scales shown in Table 2. Analysis of variance techniques were applied to individual panelist scores using cooking pressure and cooling rate as factors (GENSTAT 5 reported by Payne et al., 1987).

RESULTS & DISCUSSION

TYPICAL PRESSURE-TIME PROFILES for each of the four cooking/cooling treatments in both studies are shown in Fig. 2(a-d). The mean initial weight, heating and cooking time to 80°C, maximum temperature, cooling time from the point where 80°C was first attained to 30°C, and weight loss expressed as a percentage of the initial weight are given in Table 3. In both studies there were no significant differences between the initial breast weights used for each of the four different cooking/cooling treatments.

Heating times and product temperatures

The mean temperature-time profiles achieved using the four cooking/cooling treatments are shown in Fig. 3(a-d). In the first study there was a considerable rise in temperature above 80°C during slow cooling; this was reduced to no more than 5°C in the second study.

Heating times to 80°C (or maximum temperature if 80°C was not achieved) did not vary between treatments in the first study. In the second, breasts heated at 1.03 bar required slightly less time (2.1 min) than those heated at 0.345 bar. Yingst et al. (1971) indicated with steam cooking, pressure affected the heating time of chicken parts to a given temperature. Our results did not show this conclusively, and the difference can be explained by the dissimilar apparatus and techniques. In general, autoclaves do not have a facility for producing a vacuum, and hence it is unlikely that the autoclave used in Yingst et al. was evacuated before heating. Conditions during cooking would have therefore included air and steam. McAdams (1954) showed increased concentration of air reduced the heat transfer coefficient during condensation; the effect of different steam temperatures was slight in comparison.

To indicate differences in severity of the cooking/cooling treatments, typical plots of maximum breast temperature are shown for both studies in Fig. 4(a-b). Points shown correspond to thermocouple positions on the probe, and plots have been produced by making the position of minimum internal temperature common to each plot for all treatments. In the first study high pressure cooking gave higher internal temperatures for both fast and slow cooling treatments. Similarly, slow cooling caused higher internal temperatures irrespective of cooking pressure. In the second study, the internal temperatures did not differ much, but at the very edge of the breast the higher cooking pressure resulted in a higher temperature.

Cooling times

During fast cooling, the time from 80 to 30°C for both studies ranged from 9.9 to 13.2 minutes. During slow cooling, the times from 80 to 30°C were considerably greater in the first study (Table 3), but the cooling time from the point where 80°C was achieved on the downward part of the temperature-time profile ranged from 8.2 to 14.2 minutes.

The cooling time from the point where 80°C was first achieved

Table 4—Mean sensory panel scores for chicken breasts cooked by steam at either 0.345 bar (5 psi) or 1.03 bar (15 psi) and cooled by either a fast or slow vacuum method[a]

	Sensory appreciation scales					Hedonic scales	
				Flavor intensity			
	Color	Tenderness	Juiciness	Chicken	Foreign	Flavor liking	Overall acceptability
Study 1							
Cooking pressure (psi)/ Cooling rate							
5/fast	−2.10	1.56	1.91	0.14	0.22	0.94	0.88
5/slow	−1.25	2.02	1.24	1.23	0.26	1.63	1.64
15/fast	−1.40	1.93	1.33	−0.04	0.15	1.27	1.46
15/slow	−0.20	2.55	0.74	2.77	0.10	3.66	3.43
(s.e.)	(0.37)	(1.01)	(0.16)	(0.44)	(0.07)	(0.40)	(0.54)
(s.e.) b	(0.19)	(0.54)	(0.20)	(0.53)	(0.07)	(0.43)	(0.39)
Differences							
5psi–15psi cooking	−0.87*	−0.45	+0.54**	−0.81*	+0.12	−1.18**	−1.18*
(s.e.)	(0.34)	(0.93)	(0.08)	(0.23)	(0.10)	(0.27)	(0.46)
N = 7 ; d.f. = 6							
fast-slow cooling	−1.02**	−0.54	+0.63**	−2.08**	+0.01	−1.54**	−1.36**
(s.e.)	(0.14)	(0.38)	(0.14)	(0.37)	(0.05)	(0.30)	(0.28)
N = 14 ; d.f. = 12							
Study 2							
Cooking pressure (psi)/ Cooling rate							
5/fast	−1.11	3.11	1.24	0.00	0.34	0.26	0.51
5/slow	−2.29	3.06	1.40	−0.43	0.26	0.54	0.63
15/fast	−1.17	2.91	1.19	0.66	0.27	1.20	1.06
15/slow	−1.29	2.69	0.91	0.00	0.24	0.97	0.91
(s.e.)	(0.55)	(0.48)	(0.08)	(0.37)	(0.08)	(0.26)	(0.28)
(s.e.) b	(0.38)	(0.33)	(0.08)	(0.48)	(0.07)	(0.35)	(0.35)
Differences							
5psi–15psi cooking	−0.47	+0.29	+0.27**	−0.54*	−0.04	−0.69**	−0.41*
(s.e.)	(0.48)	(0.41)	(0.06)	(0.15)	(0.06)	(0.09)	(0.13)
N = 7 ; d.f. = 6							
fast-slow cooling	+0.64*	+0.14	+0.06	+0.54	+0.06	−0.03	+0.01
(s.e.)	(0.27)	(0.23)	(0.05)	(0.34)	(0.05)	(0.25)	(0.25)
N = 14; d.f. = 12							

[a] Scales: See Table 2.
[b] For comparison of means with same cooking pressure
* Significantly different at 5% level
** Significantly different at 1% level

to 30°C was significantly ($p < 0.05$) affected by pressure release rate in both studies, the mean difference in cooling time (fast < slow) being 21.0 minutes for the first and 5.3 minutes for the second study. The greater cooling time in the first study for slow pressure release rate was caused by the excess temperature, as shown in Fig. 3c and 3d.

The cooling times can be compared with conventional air chilling. Everington and Sagoo (1986) reported on the cooling of cooked chicken legs from 34°C in a high velocity air chilling system, and found that cooling times to 5°C exceeded 40 minutes. The longer cooling times associated with such a method increase the possibility of spoilage organisms multiplying during the critical period between the high cooking temperature and low chilled temperature.

Weight loss

The overall weight loss was affected by both cooking pressure and cooling rate in the first study, but only by cooling rate in the second. The range of weight loss in the first study, 26.4 to 37.7%, was much greater than in the second, 28.5 to 31.5%, due to the higher internal temperatures during slow cooling.

The weight losses were much higher than those reported by Yingst et al. (1971), and Klose and Mercuri (1973) when cooking chicken at similar pressures. The difference probably was due to loss of moisture from the product during vacuum cooling. Although not measured directly, the magnitude of this moisture loss can be estimated from the difference between overall weight loss for pressure cooking/vacuum cooling in our results, and the overall losses reported by Klose and Mercuri for pressure cocking only. Such an estimate gives a moisture loss during vacuum cooling of about 1.2% per 7°C reduction in temperature, slightly higher than the 1% per 7°C reported by Hofmans anc Veerkamp during vacuum chilling of chicken carcasses. The difference may be due to experimental error, but can be partially explained by reduction in latent heat of evaporation of water at higher temperatures.

Everington and Sagoo (1986) report a weight loss of 0.1% per 7°C reduction for cooling of cooked chicken in air. Thus, the moisture loss associated with vacuum cooling was an order of magnitude higher.

Sensory evaluation

The mean sensory panel scores for both studies are given in Table 4.

In both experiments neither cooking pressure nor rate of pressure release had any significant effect on tenderness of the chicken breast. The disintegration previously found when cooking whole poultry, particularly turkey at higher pressures, was not apparent in the chicken breasts in our studies. This was probably because of much shorter temperature/time treatments. All of the cooked/cooled poultry was tender and this may have masked evidence of rate of pressure release affecting tenderness.

Significant differences in juiciness between treatments have always been reported where there were significant differences in weight loss. Linear regression of juiciness on weight loss revealed a correlation coefficient, $r = -0.72$.

—Continued on page 1551

Tocopherols in Chicken Breast and Leg Muscles Determined by Reverse Phase Liquid Chromatography

C. Y. W. ANG, G. K. SEARCY, and R. R. EITENMILLER

ABSTRACT

Tocopherols were extracted from broiler muscles with hexane after saponification and determined by reverse phase liquid chromatography with fluorescence detection. Both alpha- and gamma- tocopherols were found in both breast and leg muscles, raw or cooked. Mean recovery values were alpha- 91.2% and gamma-tocopherols 94.2%. Leg meat contained almost twice as much total tocopherols as breast meat. This favorable distribution of tocopherols may be significant in retarding oxidative changes of cooked leg tissues during post-cooking storage. Cooking had no significant effect on retention of tocopherols on a dry weight basis for breast or leg meat.

INTRODUCTION

VITAMIN E ACTIVITY in food is derived from a series of compounds of plant origin, the tocopherols and tocotrienols. Due to difficulty in analytical methodology, especially for meat tissues, information on composition of tocopherols in poultry meat is scarce. The USDA Agriculture Handbook No. 8-5 (CFEI, 1979) includes the values of nine vitamins in various poultry products, but no information on vitamin E.

Only limited data on vitamin E content of chicken have been reported. In compiling published data, McLaughlin and Weihrauch (1979) noted that for raw muscles from mammals and birds, vitamin E was nearly all in the form of alpha-tocopherol; only small amounts of other tocopherols were found in a few samples. No tocotrienols were found. Specific analytical methods were not reported since the data were compiled from various laboratories and different methodologies probably were used.

In recent years, with the advent of high performance liquid chromatography (HPLC), various forms of tocopherol in meat tissues could be determined precisely at low levels (Parrish, 1980). However, data are still limited. From one pooled sample of raw chicken flesh, Piironen et al. (1985) reported that alpha-tocopherol was the primary form (0.7 mg/100g) and that gamma-tocopherol was much lower (0.06 mg/100g). However, Lehmann et al. (1986) reported that roasted chicken breast meat (2 samples) contained gamma-tocopherol (0.09–0.14 mg/100g) that was not much lower than the alpha-tocopherol (0.15-0.18 mg/100 g). The presence of gamma-tocopherol in chicken is of interest to food technologists because this form also is an effective antioxidant, though the alpha form is more potent in vitamin E activity than other forms. The natural distribution of various tocopherols in different muscle tissues would affect the storage stability of processed poultry products.

The objectives of our study were to determine the level of different forms of tocopherol in broiler chicken breast and leg muscles using a reverse phase HPLC method and to assess the effect of cooking treatment on the stability of these tocopherols.

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MATERIALS & METHODS

Broiler muscle samples

Fresh ready-to-cook broiler chickens without giblets were obtained from local processing plants or supermarkets. They were stored at 4°C and used within 3 days. Each carcass was cut into two halves. The skin, bones and visible fatty tissues were removed with a small knife. Each half was separated into two parts: breast meat and leg meat (thigh and drum stick). Samples were placed in heat-sealable plastic bags and stored at −10°C. Each bag contained one type muscle (about 90–120g) from one half broiler. Samples from the left sides of the birds were used for raw meat analysis and samples from the right sides were used for cooked meat analysis. Three broilers were used in each independent lot, i.e., collection of broilers, preparation, cooking treatment and analysis. Four lots were analyzed at different months throughout the year.

Cooking treatment

Frozen parts in sealed bags were cooked in 85–87°C water bath for about 30 min to internal temperature 80–82°C in the center of the thickest part of the muscle. Preliminary tests were conducted to determine cooking time. Internal temperature of the cooked sample was measured with a hand-held electronic digital thermometer with a tubular point metal probe (Fisher Scientific Co., Norcross, GA). After cooking, sample bags were immediately cooled in ice water 10 min which reduced the internal temperature to about 10°C. Samples were removed from the bags and solidified cooking juice was scraped off the meat surface.

Sample preparation and dry weight

Cooked muscles (entire sample from a bag) were blended in a food processor (Hamilton Beach, Model 702-1, Washington, NC) 15 sec under nitrogen. Frozen raw meat was thawed at 4°C for 2–4 hr prior to blending. Aliquots of 2–3g blended samples were accurately weighed in disposable aluminum weighing dishes and dried in an oven at 95°C for 4 hr without vacuum, followed by vacuum drying an additional 48 hr. Duplicate analyses were made on each sample.

Chemicals and reagents

Reference standards of alpha-, beta- and gamma-tocopherol were obtained from Eastman Kodak Co. (Rochester, NY) or the Henkel Corp. (LaGrange, IL). They were ≥ 98% pure. Standard stock solutions were prepared at 100 mg/100 mL in methanol and intermediate standards at 2 mg/100 mL. Mixed working standard solutions, five levels ranging from 0.0125 to 0.2 mg/100 mL, were made by appropriate dilutions of the intermediate solutions with methanol. All standards were stored in low actinic glassware and experiments were carried out under yellow fluorescent lights to protect tocopherols from changes due to light exposure. All reagents for HPLC separations were HPLC grade and all other chemicals and reagents were analytical grades. Distilled, deionized water was used throughout and all mobile phase solutions were filtered through a 0.45 μm Millipore HA membrane filter and degassed prior to use. All blank reagents were checked to confirm absence of background peaks.

Saponification and extraction of tocopherols

The saponification and extraction procedures were adapted from Piironen et al. (1985). Samples were analyzed in duplicate or triplicate. Sample size was 2.5g. Ascorbic acid (0.25g), distilled water (5 mL) and 100% ethanol (12.5 mL) were added to the sample test tube,

which was then mixed for 10–15 sec on a Vortex mixer followed by standing for 15–20 min prior to addition of 3 mL 50% KOH solution. The tube was flushed with nitrogen, screw-capped and further mixed 15-20 sec. All tubes were placed in a shaker water bath at 30°C and held overnight followed by 2 hr at 50°C. After cooling in a cold water bath, the saponified sample mixture was rinsed into a separatory funnel with 30 mL of 50% ethanol and extracted three times with 30 mL hexane. The combined hexane extract containing tocopherols and other unsaponifiables was washed with water, evaporated to dryness at 50°C under vacuum and redissolved in 10 mL methanol. The methanol solution was filtered through a 0.45 μm membrane prior to injection in the LC system.

High performance liquid chromatography

The instrument system included a Model 6000A Solvent Delivery System (Waters Associates, Milford, MA), a Rheodyne Model 7125 injector with a 20-μl fixed volume loop, a Perkin Elmer Model 650 fluorescence detector (Perkin Elmer Corp., Norwalk, CT), Model 730 Data Module and Model 720 System Controller (Waters Associates). The detector excitation wavelength was 296 nm and emission was 330 nm.

The separation and analysis of tocopherols were carried out on a C_{18} reverse phase BioSil ODS-5S column, 250 mm x 4 mm I.D. (BioRad Laboratories, Richmond, CA) with 100% methanol as the mobile phase (Shaikh et al., 1977) at 1 mL/min.

The identification of each form was made by matching the retention time of the unknown peak with that of the standard compound, by adding the standards to the samples before saponification and verifying the retention time of specific peaks of interest. Detector relative response tests (Snyder and Kirkland, 1979) were run to further confirm peak purity. The test compares the ratio of detector responses of an unknown peak at two conditions (two sets of wavelengths) with the ratio of a standard at the same conditions. The identity of an unknown peak is confirmed if its detector response ratio is the same as that of the standard.

The excitation wavelengths used for the tests were 275 and 280 nm with the emission wavelength set at constant (330 nm). The detector response ratios determined were: 0.58, at 275 and 1.10 at 280 nm, for the alpha-tocopherol standard and 0.60 at 275 and 1.13 at 280 for the sample alpha peak; 0.33 at 275 and 0.65 at 280 for gamma-tocopherol standard and 0.33 at 275 and 0.66 at 280 for the sample gamma peak.

Standard solutions were analyzed on the same day as experimental samples. Standard curves were constructed and concentrations of tocopherols in sample extracts were computed from the standard curves. For recovery tests, mixed standard solutions were added to sample aliquots prior to saponification. In preliminary tests, standard solutions alone were saponified, extracted and analyzed following the same procedures.

Statistical analysis

Statistical computations were run on the Statistical Analysis System (SAS, 1985). The differences in tocopherol content between cooked and raw muscles were tested by the paired comparison procedure (TTEST Procedure).

RESULTS & DISCUSSION

TWO MAJOR PEAKS, alpha- and gamma-tocopherols, were found in all samples. Typical chromatograms of the muscle extracts are shown in Fig. 1. Detection of the gamma-tocopherol attracted our special attention, since only limited published data indicated its presence in cooked chicken (Lehmann et al., 1986). Further identification of this peak was made by: (1) reverse phase HPLC with 95% methanol as the mobile phase (Shaikh et al., 1977), (2) reverse phase HPLC with acetonitrile: methylene chloride: methanol (70:30:5, v/v/v) as the mobile phase (Landen, 1982), (3) normal phase HPLC with 1% isopropanol in hexane as the mobile phase (Hakansson et al., 1987), and (4) gas chromatography via propionate esters (Eastman Kodak, 1986). Some of the confirmatory analyses were conducted in other laboratories by collaborators. The peak in question was concluded to be gamma-tocopherol. A trace of beta-tocopherol but no alpha-tocotrienol was identified by the normal phase system. Due to the extreme low level of beta isomer, no attempt was made to quantify it.

The average and standard deviation of recoveries of standards in reagent blanks of 12 analyses through the complete saponification, extraction and HPLC procedures were 93.5 ± 2.8% for alpha- and 92.2 ± 2.6% for gamma-tocopherols. Samples had added 0.08–0.13 mg/100g tocopherols for recovery tests. Average ± standard deviation recoveries from 12 samples (3 added samples per cooking state per type of muscle tissues) were 91.2 ± 8.0% for alpha and 94.2 ± 3.0% for gamma-tocopherols. No correction factors were applied for calibration.

Tocopherols content for raw and cooked muscles is shown in Tables 1 and 2, respectively. The ratios of distribution of the two forms of tocopherols between leg and breast tissues are also presented. Wide variations in tocopherol content were observed among broilers, but significant differences were found between the two parts ($P < 0.01$). Leg meat contained more of each form than the breast meat ($P < 0.01$). However, regardless of variations among animals, ratios of distribution in the leg and breast tissues were nearly constant.

McLaughlin and Weihrauch (1979) reported an average of 0.34 mg/100 g of total vitamin E and 0.29 mg/100 g of alpha-tocopherol from 23 raw chicken samples; 0.55mg/100g total vitamin E and 0.35 mg/100g of alpha-tocopherol from 3 cooked chicken samples. Lehmann et al. (1986) reported significant amounts of gamma-tocopherol (0.09-0.14mg/100g) as well as alpha-tocopherol (0.15-0.18 mg/100 g) in roasted chicken breast meat from two samples. Our results differ slightly from data presented by McLaughlin and Weihrauch (1979) on alpha-tocopherol content and agree with results of Lehmann et al. (1986) regarding gamma form content in cooked breast meat. However, our study determined a lower level of alpha-tocopherol content than those reported by Piironen et al. (1985), who determined 0.70 mg/100 g of alpha-tocopherol, with much lesser amounts of tocotrienols and gamma-tocopherol (0.03-0.06 mg/100 g) in raw chicken flesh. Their data were from one pooled sample. If samples were analyzed individually, variations of the tocopherol content most likely would have been evident.

Since cooked leg meat contains more iron and phospholipids than breast muscle (Ang, 1988), it is possible that leg meat underwent faster oxidative changes than the breast meat. However, our previous data (Ang, 1988; Ang and Lyon, 1990; Ang, et al., 1989) did not consistently support that hypothesis. We found that cooked, stored leg meat did not always oxidize faster or develop more off-flavors than breast meat treated in the same manner. Thus, we hypothesized that leg meat contained more total tocopherols, a group of natural antioxidants, than breast meat. Only one study (Yamauchi et al., 1982) showed the alpha-tocopherol content was higher in dark muscle than in light muscle. Yamauchi, et al. (1982) also reported that the TBA numbers were not always higher in the cooked, stored dark meat than the light meat. However, they determined only the alpha-tocopherol using a thin layer chromatographic method.

Several other reports have shown that vitamin E supplement (alpha-tocopherol acetate) was effective in reducing lipid oxidation in poultry meat and preventing off-flavor development as measured by the thiobarbituric acid (TBA) method and sensory scores (Bartov et al., 1983; Marusich et al., 1975). Results of our study provide a base for explaining how leg meat may be protected from oxidative changes and flavor deteriorations during post-cooking storage.

In our study, paired comparisons were made between raw and cooked meat of the same bird. The effect of cooking on retention of tocopherols was determined independently from the variation of animals. On a wet weight basis, the cooked meat contained somewhat higher amounts of tocopherols than the raw meat. However, that was mainly due to dilution effect;

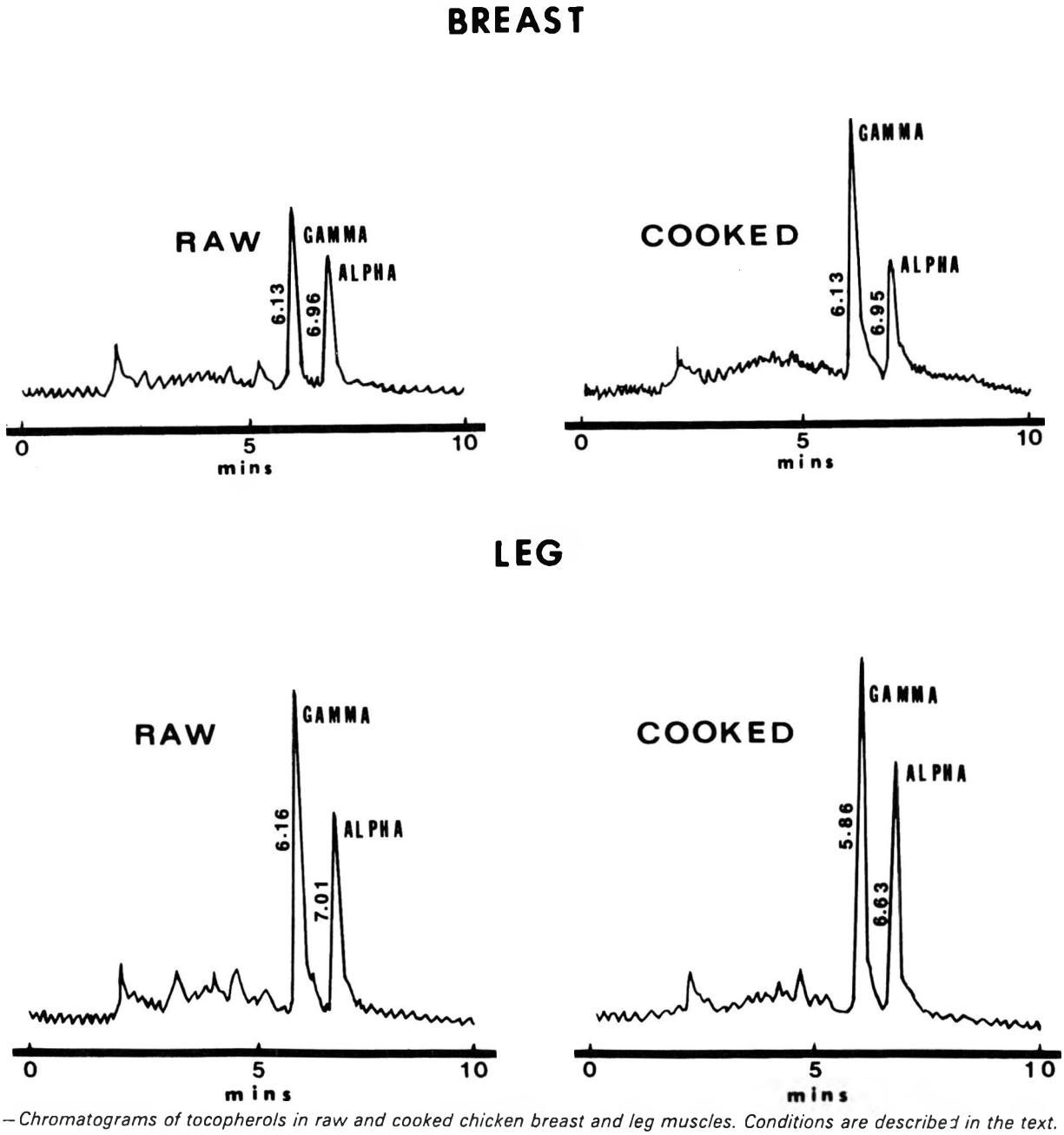

Fig. 1—Chromatograms of tocopherols in raw and cooked chicken breast and leg muscles. Conditions are described in the text.

Table 1—Tocopherols content of raw broiler chicken breast and leg muscles (mg/100g wet weight)

Lot no.	Breast			Leg			Ratio of total forms (leg/breast)
	Alpha	Gamma	Total	Alpha	Gamma	Total	
1	0.19[a]	0.08	0.27	0.35	0.15	0.50	1.9
	(0.07)	(0.04)	(0.11)	(0.10)	(0.07)	(0.17)	(0.2)
2	0.23	0.11	0.34	0.42	0.19	0.61	1.8
	(0.03)	(0.01)	(0.04)	(0.09)	(0.05)	(0.09)	(0.2)
3	0.17	0.10	0.27	0.41	0.18	0.58	2.2
	(0.08)	(0.01)	(0.09)	(0.11)	(0.03)	(0.14)	(0.06)
4	0.19	0.10	0.29	0.42	0.19	0.61	2.1
	(0.02)	(0.02)	(0.09)	(0.01)	(0.03)	(0.04)	(0.1)
Mean	0.20	0.10	0.29	0.40	0.18	0.58	2.0
Std Dev (N=12)	0.05	0.02	0.07	0.08	0.04	0.11	0.3

[a] Mean (standard deviation) of 3 broilers per lot.

Table 2—Tocopherols content of cooked broiler chicken breast and leg muscles (mg/100 g wet weight)

Lot no.	Breast			Leg			Ratio of total forms (leg/breast)
	Alpha	Gamma	Total	Alpha	Gamma	Total	
1	0.20[a]	0.08	0.28	0.33	0.17	0.50	1.8
	(0.06)	(0.04)	(0.09)	(0.13)	(0.10)	(0.23)	(0.3)
2	0.24	0.12	0.36	0.51	0.22	0.73	2.0
	(0.10)	(0.02)	(0.08)	(0.03)	(0.01)	(0.04)	(0.1)
3	0.21	0.12	0.33	0.40	0.21	0.61	1.8
	(0.02)	(0.01)	(0.02)	(0.02)	(0.02)	(0.01)	(0.1)
4	0.20	0.15	0.35	0.48	0.22	0.70	2.1
	(0.01)	(0.02)	(0.03)	(0.09)	(0.05)	(0.13)	(0.5)
Mean	0.21	0.12	0.33	0.43	0.21	0.64	1.9
Std Dev (N=12)	0.03	0.03	0.06	0.10	0.05	0.15	0.3

[a] Mean (standard deviation) of 3 broilers per lot.

cooked meats contained more dry matter than raw meat (Table 3). On a dry weight basis, the changes in tocopherol content during cooking, over 12 broilers of 4 lots, were not significant (Table 3).

Data from our study provide basic information on the distribution of two forms of tocopherol in two broiler muscles. The effect of cooking on retention of alpha and gamma forms in breast and leg meats were further determined. The infor-

Table 3—Retention of tocopherols in broiler chicken breast and leg muscles during cooking (% retention dry weight basis)

Lot No.	Muscle type	Water (%) Raw	Water (%) Cooked	Tocopherol (% retention) Alpha	Gamma	Total
1	Breast	74.35[a]	70.86	91.8	95.2	93.5
2	Breast	74.20	71.91	100.0	98.6	99.3
3	Breast	75.20	71.49	117.7	107.6	112.5
4	Breast	75.61	71.68	99.4	116.9	108.1
Mean (N = 12)		74.84	71.48	99.8	109.6	104.7
Std Dev		0.98	0.60	24.1	25.1	17.4
				NS[b]	NS	NS
1	Leg	75.12	69.05	74.8	86.6	80.7
2	Leg	74.84	71.14	107.8	101.2	103.2
3	Leg	76.38	70.73	83.8	98.0	90.8
4	Leg	76.83	71.75	94.7	95.5	95.1
Mean (N = 12)		75.79	70.67	90.3	95.4	92.9
Std Dev		0.94	1.24	22.1	10.0	15.1
				NS	NS	NS

[a] Average of 3 broilers per lot.

[b] No significant difference between cooked and raw samples.

mation will be used for further research concerning the mechanisms of oxidative stability of processed poultry products.

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Thermal Transitions of Salt-soluble Proteins from Pre- and Postrigor Chicken Muscles

Y.L. XIONG and C.J. BREKKE

ABSTRACT

Salt-soluble protein (SSP) was extracted from pre- and postrigor chicken muscles at various pH values, and protein thermal denaturation was studied using several techniques. Heating at 1°C/min from 20 to 70°C induced a three- to fourfold increase in breast and leg hydrophobicity. Differential scanning calorimetry of breast and leg SSP showed a major transition occurring within the range 55 to 64°C, with the value dependent on rigor state and pH. Protein-protein association, as measured by turbidity change upon heating, underwent two transitions for leg SSP and two or three for breast SSP. The specific transition temperature and rate were dependent on pH, muscle type and rigor state. However, muscle type and pH had a greater effect than muscle rigor state on SSP denaturation.

INTRODUCTION

THE BINDING of meat pieces in restructured and formed or other comminuted meat products results from thermal denaturation of salt-extracted myofibrillar proteins (MacFarlane et al., 1977; Siegel and Schmidt, 1979). These proteins, collectively called salt-soluble protein (SSP), can vary in composition, depending on concentration of NaCl or ionic strength and pH of the extraction buffer (Foegeding, 1987). Furthermore, SSP extracted from postmortem muscles in various rigor states contains different protein components, i.e., myosin, actin and actomyosin complex (Sayre, 1968).

Among other environmental conditions, pH is an important factor influencing thermally induced protein changes. Thermal transitions of myosin and actomyosin are dramatically influenced by pH (Goodno and Swenson, 1975; Wright et al., 1977; Samejima et al., 1983). The thermal denaturation of myofibrillar tissues is also affected by pH (Stabursvik and Martens, 1980; Wagner and Anon, 1985).

Various techniques are used to determine protein unfolding and interaction during denaturation, including differential scanning calorimetry (DSC), hydrophobicity change and turbidity change of the protein suspension caused by protein-protein aggregation. DSC has been widely used to study muscle protein denaturation (Wright et al., 1977; Stabursvik and Martens, 1980; Samejima et al., 1983; Xiong et al., 1987), but DSC generally requires high muscle protein concentration. Li-Chan et al. (1985) succeeded in determining thermally induced protein unfolding by measuring protein hydrophobicity changes with a fluorescence probe, 8-anilino-1-naphthalene sulfonate (ANS). ANS fluorescence intensity increases when a more nonpolar (hydrophobic) region of a protein becomes available as a result of protein unfolding. On the other hand, Ziegler and Acton (1984) and Acton and Dick (1986) studied thermally induced protein- protein interactions by continuously monitoring changes in optical density of actomyosin solution upon heating. These authors showed aggregation of proteins during denaturation resulted in increasing turbidity of the solution.

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The objective of our investigation was to study the thermal denaturation of SSP from pre- and postrigor chicken breast and leg muscles using hydrophobicity, DSC and turbidity techniques, and to determine how thermal transitions were affected by pH of protein suspensions.

MATERIALS & METHODS

Muscle source and preparation

Live white Leghorn hens (age 17–18 months) were purchased from a local poultry farm. In each replication, breast and leg muscles from two chickens, sacrificed at the same time, were pooled as muscle samples. After 2 min bleeding, the right breast (pectoralis major and minor combined) and leg (thighs and drumsticks combined) muscles were excised and chilled in crushed ice 5 min. External fat and heavy connective tissue were removed, and the diced muscles ground for myofibril isolation. Total elapsed time between death and myofibril isolation was ≤ 25 min.

The unboned left breast and leg were used as the postrigor muscle sample source. The breast and legs, with skin removed, were excised from the carcass immediately after bleeding and chilled in crushed ice about 30 min. The breast and legs were wrapped in paper towels moistened with 1 mM sodium azide (NaN_3), packed in 3 mil polyethylene plastic bags, and stored in ice slurries 24 hr prior to myofibril isolation.

Salt-soluble protein (SSP) preparation

SSP was extracted from myofibrils prepared from pre- and postrigor muscles as previously described (Xiong and Brekke, 1989). The extraction buffers contained 50 mM piperazine-N,N bis (2-ethane sulfonic acid) (PIPES), 0.6M NaCl and 1 mM NaN_3 at various pH values.

Concentration of protein was determined by the biuret method (Gornall et al., 1949) using bovine serum albumin as the standard.

Hydrophobicity

SSP was suspended and adjusted to 0.1 mg/mL in 50 mM PIPES buffer containing 0.6M NaCl and 1 mM NaN_3 at specific pH values. The SSP suspensions were heated at 1°C/min from 20 to 70°C in a water bath. After reaching specific temperatures, duplicate samples were removed from the bath, cooled in ice slurries, and stored in ice 3 hr before analysis.

Thermally induced unfolding of protein structure was determined according to the method developed by Li-Chan et al. (1985) using 8-anilino-1-naphthalene sulfonate (ANS) as the fluorescence probe. Fluorescence was detected with an Aminco-Bowman Spectrophotofluorometer (J4-8961) equipped with a photomultiplier microphotometer (J10-280) (American Instrument Co., Division of Travenol Lab., Inc., Silver Spring, MD). 10 µL ANS (8.0 mM in 50 mM PIPES, pH 6.00) were added to 2 mL protein solutions. The relative fluorescent intensity was determined 45 min after ANS addition. ANS-protein conjugates were excited at 390 nm, and the fluorescence emitted at 468 nm; the excitation and emission slits were 1 and 2 nm, respectively. "Protein blank" fluorescence values were measured in absence of ANS and were subtracted from the sample (with ANS added) fluorescence values. The fluorescent intensity was used to represent hydrophobicity (unfolding) of the proteins.

Differential scanning calorimetry

DSC was conducted in a Perkin-Elmer DSC-4 fitted with a 3600 Thermal Analysis Data Station (Perkin-Elmer Corp., Norwalk, CT). SSP samples (55 - 60 mg) were hermetically sealed in stainless steel

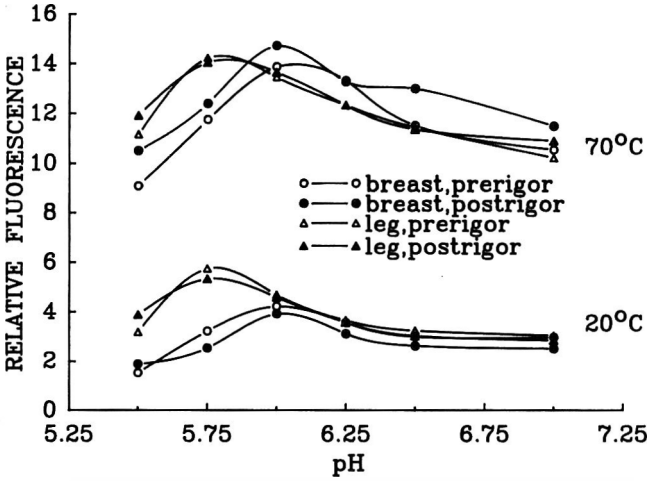

Fig. 1—Effect of pH and temperature on hydrophobicity (fluorescence intensity) of SSP from pre- and postrigor chicken breast and leg muscles. Means of three replicates.

large-volume capsules (75 μL volume) and thermally scanned at 10°C/min over 20 to 100°C. The instrument was temperature-calibrated using indium (m.p. 156.60°C). The maximum thermal transition temperatures (Tm) were determined with data analysis programs supplied by Perkin-Elmer. A stainless steel capsule containing 60 mg water was used as reference.

Protein-protein interaction

SSP was suspended and adjusted to 0.3 mg/mL in 0.6M NaCl, 50 mM PIPES and 1 mM NaN_3 at pH 5.50, 6.00 and 6.50. Thermally induced protein-protein association/interaction was determined by monitoring turbidity change of the protein solution during heating (Acton and Dick, 1986). The protein solution was heated at 1°C/min from 20 to 75°C; increasing turbidity of the solution was recorded each minute by measuring changes in optical density at 320 nm in a Perkin-Elmer UV-VIS spectrophotometer (Model Lambda 5) equipped with a temperature-controlled water jacket (Perkin-Elmer Corp., Norwalk, CT). Temperature of samples was monitored with a thermocouple. The differential change in optical density as a function of heating temperature (dA_{320}/dT) was calculated to determine rate and transition temperature of the protein-protein interaction.

Statistical analysis

In our study, replications are defined as the experimental work from different protein (SSP) preparations at different times. In each replication, two chickens were used, and the breast or leg muscles were pooled for SSP extraction.

The thermal transition temperatures (Tm) from duplicate or triplicate measurements from different replicate trials were averaged to obtain mean values for each specific transition. Difference between means was determined by the Student's t-test (Steel and Torrie, 1960).

RESULTS & DISCUSSION

SSP-ANS fluorescence

The effect of pH on the hydrophobicity of SSP was determined by measuring fluorescence intensity (Fig. 1). Heating from 20 to 70°C caused a three- to fourfold increase in hydrophobicity, suggesting an unfolding of protein with more hydrophobic or non-polar regions of proteins being thermally exposed on heat application. However, breast SSP had a different pH dependence than leg SSP for the observed hydrophobicity. Breast SSP-ANS conjugate fluoresced with the greatest intensity at pH 6.00, whereas for leg, the maximum fluorescence of the SSP-ANS conjugate was at pH 5.75. The dependence of protein fluorescence on pH was likely induced by protein conformational changes caused by the altered intra- and/or intermolecular electrostatic interactions.

The fundamental role of pH in protein conformation is well established (Cheftel and Cuq, 1985). When pH approached the isoelectric point (pI) of the SSP (5.00–5.40), the net charge density would have approached zero, reducing electrostatic repulsion and facilitating protein aggregation. The decrease in hydrophobicity of SSP when pH was reduced from 6.00 to 5.50 suggests a reduction of exposure of hydrophobic regions or a decrease in accessibility of hydrophobic groups which may be involved in aggregation. On the other hand, the decrease in hydrophobicity of either breast or leg SSP as pH increased may have resulted from increased protein-protein repulsion due to increased net charge density, presumably from deprotonation of histidine. A more polar environment would allow fewer hydrophobic groups to be exposed. Since the quantum yield of ANS fluorescence probe is insensitive to pH in the range of 2-8 (Gibrat and Grignon, 1982), the pH effect on hydrophobicity as shown by the protein-ANS fluorescence in our study, apparantly was due mainly to changes in protein unfolding and protein-protein interactions.

The hydrophobicity of neither breast nor leg SSP was dependent on muscle rigor state (Fig. 1). The composition of the SSP, whether myosin and actin were associated or free from each other, would not seem to significantly affect SSP hydrophobicity.

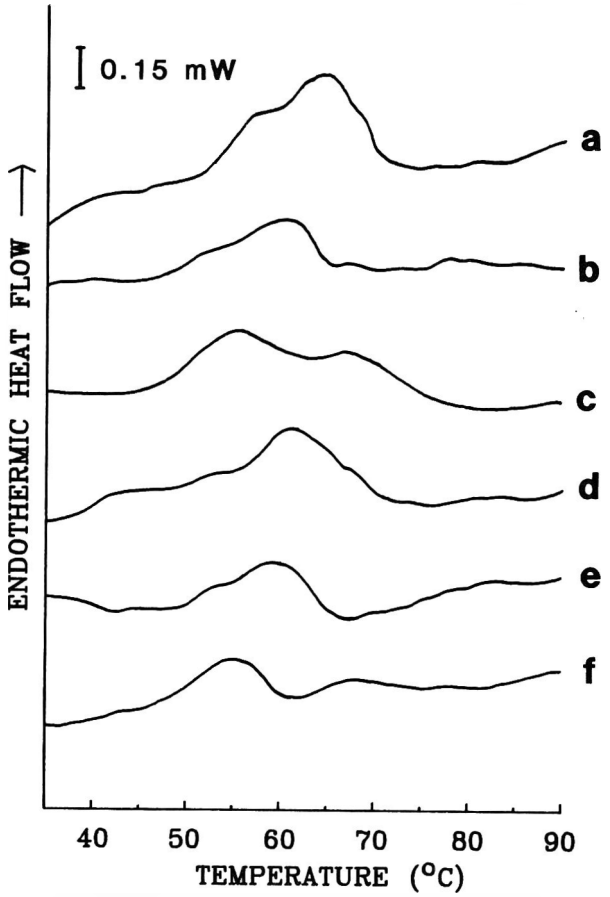

Fig. 2—DSC thermal curves of SSP extracted from pre- and postrugir breast muscle tissue at different pH. SSP contained 0.1 M NaCl, 8 mM PIPES and 30–35 mg/mL protein. Thermal scan rate = 10°C/min. Curves a, b and c = postrigor SSP extracted at pH 6.50, 6.00 and 5.50, respectively; curves d, e and f = prerigor SSP extracted at pH 6.50, 6.00 and 5.50, respectively.

Differential scanning calorimetry

The effects of pH and muscle rigor state on thermally induced denaturation of SSP are shown in Fig. 2, Fig. 3 and in Table 1. All SSP samples, except breast SSP at pH 5.50,

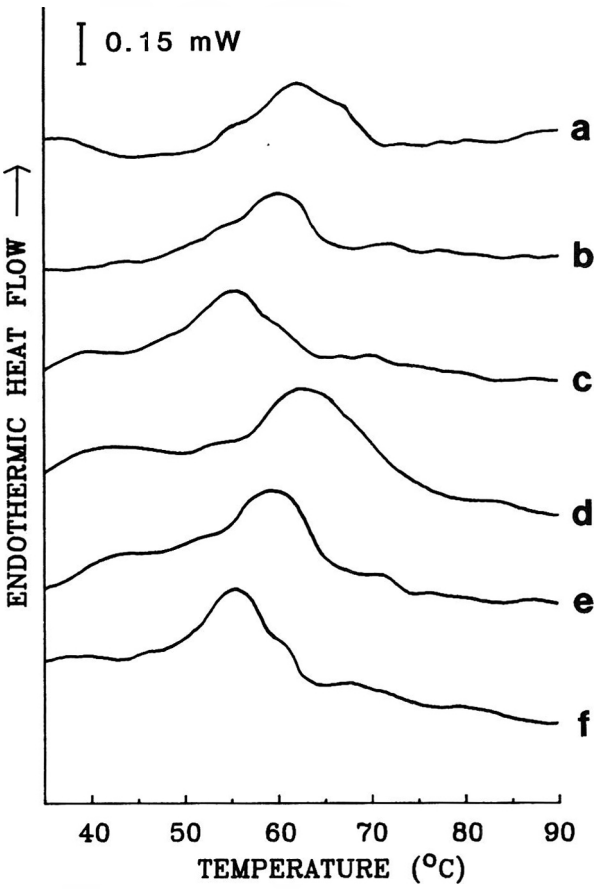

Fig. 3—DSC thermal curves of SSP extracted from pre- and postrigor leg muscle tissue at different pH. SSP samples contained 0.1 M NaCl, 8 mM PIPES and 30–35 mg/mL protein. Thermal scan rate = 10°C/min. Curves a, b and c = postrigor SSP extracted at pH 6.50, 6.00, and 5.50, respectively; curves d, e and f = prerigor SSP extracted at pH 6.50, 6.00 and 5.50, respectively.

showed a single thermal transition which ranged from 55 to 64°C but which included some unresolved "shoulders." Breast and leg, and pre- and postrigor samples, were affected similarly by pH. In general, the transition temperatures (Tm) increased with pH. Goodno and Swenson (1975) reported that lowering pH from 7.0 to 5.4 (0.5M KCl) reduced myosin Tm from 43 to 37°C. They suggested binding of hydrogen ions to myosin altered the charge on the myosin molecules and disturbed the balance between electrostatic forces that maintain native structure of protein.

As displayed in Fig. 2, at pH 5.50 pre- and postrigor breast SSP underwent two thermal transitions at 55 and 68°C. The 55°C transition may be related to myosin, but the 68° transition can be attributed to actin. Wagner and Anon (1985) showed when pH was reduced from 6.2 to 5.4, actin in myofibrils became more heat-stable, as indicated by the increased Tm, whereas myosin became more unstable. Furthermore, the number of thermal transitions of myofibrils has been shown to decrease as pH is reduced from 6.2 to 5.4 (Stabursvik and Martens, 1980; Wagner and Anon, 1985). Although Tm between pre- and postrigor breast at pH 5.50 and 6.00, were not different Tm of postrigor SSP was significantly greater ($P < 0.05$) than Tm of prerigor SSP at pH 6.50 (Table 1). Postrigor SSP was expected to have a different composition, i.e., higher actomyosin content, than prerigor SSP and, therefore, the different Tm values may be due to differences in composition.

No differences in TM were detected between pre- and postrigor leg SSP at a given pH (Table 1), although prerigor SSP components would be different from postrigor SSP. In addition, at these three pH levels, all SSP showed a single transition. These results indicated leg SSP thermal properties were considerably different from those of breast SSP. The difference may be ultimately ascribed to the isozymic forms of myfibrillar proteins, such as myosin, between red and white muscles.

Protein-protein interaction

When thermally induced interaction/association of proteins was determined by monitoring turbidity during heating (Fig. 4 to 6), leg SSP, at pH 5.50, 6.00 and 6.50, showed two thermal transitions in. The first occurred at 38, 44 and 51°C for pH 5.50, 6.00 and 6.50, respectively, and the second occurred at 49, 53 and 63°C for pH 5.50, 6.00 and 6.50 proteins, respectively (table 1). Similar results were reported by Deng et al. (1976) and Ziegler and Acton (1984) who showed aggregation of actomyosin was initiated at lower temperatures when pH was reduced. However, the rate of turbidity change, indicated by height of the peaks on derivative curves, was greater for the first transition than for the second. However, transition of leg SSP was the most abrupt and sharpest at pH 6.00, which indicated that variations existed in the mechanism of protein-protein interaction at different pH values. Thus the pH-related differences shown in the thermally induced protein-protein association are suggested as mainly due to changes in electrostatic interaction of proteins.

The pH effect on aggregation of leg protein molecules appeared to be similar to the pH effect on denaturation determined by DSC. More specifically, reducing pH destabilized protein as indicated by decrease of Tm in DSC, and decreased the temperature for aggregation. The two thermally induced transitions upon protein-protein interaction of leg SSP were presumably caused by unfolding followed by association/aggregation of myosin (peak 1) and actin (peak 2) and/or by actomyosin, since they were the major proteins in the extracted SSP samples.

At pH 5.50, postrigor breast SSP underwent a marked transition at 62°C, much more abrupt than prerigor breast SSP.

Table 1—Transition temperature (Tm) for SSP extracted from pre- and postrigor muscle myofibrils at different pH

	SSC (dH/dT)[a] (10°C/min, μ = 0.1)				Turbidity (dA_{320}/dT)[b] (1°C/min, μ = 0.6)							
pH	5.50	6.00	6.50	pH	5.50		6.00			6.50		
	Tm	Tm	Tm		Tm1	Tm2	Tm1	Tm2	Tm3	Tm1	Tm2	Tm3
Breast, prerigor	54.8	59.9	61.9[c]		46.7	62.6	42.8	52.7	60.9	45.1	51.7	62.5
	(0.9)	(0.3)	(0.8)		(0.5)	(1.1)	(0.5)	(1.3)	(1.8)	(0.1)	(1.1)	(1.3)
Breast, postrigor	55.2	60.9	63.8[c]		46.3	61.5	43.7	52.4	59.5	45.1	52.4	63.0
	(0.7)	(0.7)	(0.9)		(0.9)	(1.8)	(0.5)	(1.4)	(1.2)	(0.2)	(0.2)	(0.2)
Leg, prerigor	55.2	59.2	62.5		37.9	48.2	43.3	53.2	–	50.9	62.5	–
	(0.6)	(0.4)	(0.3)		(2.9)	(2.3)	(0.6)	(0.4)	–	(2.6)	(0.5)	–
Leg, postrigor	54.9	59.9	62.0		38.1	49.1	43.6	53.2	–	51.5	63.9	–
	(0.3)	(0.8)	(0.6)		(3.9)	(2.9)	(0.8)	(0.9)	–	(2.4)	(1.2)	–

[a] Mean values and standard deviations (in parentheses) of two replicates (2 chickens per replicate).
[b] Mean values and standard deviations (in parentheses) of four replicates (2 chickens per replicate).
[c] Significantly different from each other ($P < 0.05$).

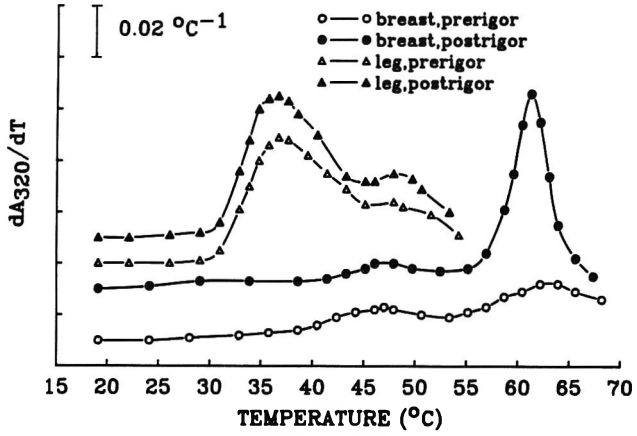

Fig. 4—Derivative curves of the protein-protein interaction at pH 5.50 for salt-soluble proteins of pre- and postrigor chicken breast and leg muscles. dA_{320}/dT = differential change in optical density as a function of temperature.

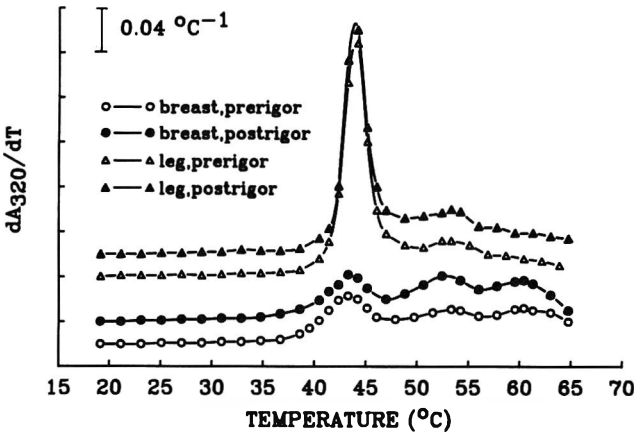

Fig. 5—Derivative curves of the protein-protein interaction at pH 6.00 for salt-soluble protein of pre- and postrigor chicken breast and leg muscles. dA_{320}/dT = differential change in optical density as a function of temperature.

This sharp transition could be ascribed to actin since it is relatively heat-stable (Stabursvik and Martens, 1980). Although the exact mechanism remains unclear, actin from postrigor muscle may have different thermal properties and energy requirements during denaturation and aggregation than actin from prerigor muscle.

In comparison to the thermal transitions at pH 5.50, breast SSP at pH 6.00 (Fig. 5) and 6.50 (Fig. 6) showed an extra transition. Basically, the SSP showed three transitions around 43, 52 and 60°C at pH 6.00, and 45, 52 and 63°C at pH 6.50 (Table 1). The first and third transitions were shifted to higher temperatures (by 2–3°C) with pH change from 6.00 to 6.50. The second transition was less sensitive to this change. The exact cause for these thermally induced transitions is not know. Based on the reported DSC analyses (Wright et al., 1977; Stabursvik and Martens, 1980), the first transition may be ascribed to myosin, the third to actin association, and the second may be attributed to myosin-actin interaction or those that also involved other myofibrillar components that comprised the SSP.

At pH 6.00–6.50, three thermally induced transitions of protein-protein interaction were recorded for breast SSP but two were observed for leg SSP. This coincides with the DSC results for chicken myofibril thermal denaturation reported earlier (Xiong et al., 1987). Three transition peaks (53, 61 and 69°C) for breast myofibrils but two (60 and 70°C) for leg myofibrils at 0.1 M NaCl, pH 7.1 were observed (Xiong et al., 1987). Such a fiber-type-related difference may correspond to isozymic forms of myosin and, perhaps, other myofibrillar proteins. Morita et al. (1987) showed chicken breast myosin molecules in salt solution interacted to form long filaments, while leg myosin molecules formed short and discrete filaments/rods.

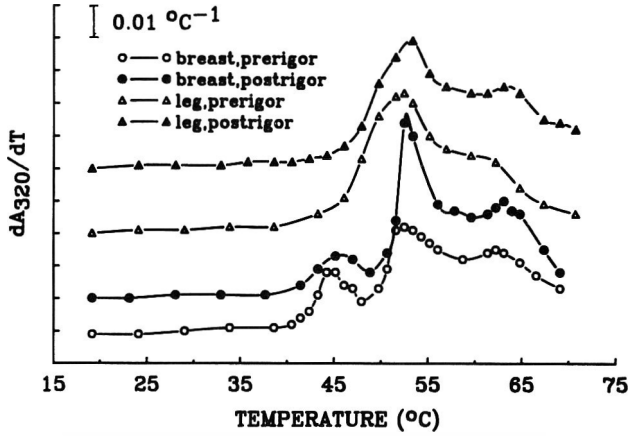

Fig. 6—Derivative curves of the protein-protein interaction at pH 6.50 for salt-soluble proteins of pre- and postrigor chicken breast and leg muscles. dA_{320}/dT = differential change in optical density as a function of temperature.

Despite discrepancies found between red and white muscle SSP and differences caused by effects of pH, the thermally induced interaction/association of SSP was generally independent of muscle rigor state except for breast SSP at pH 5.50. Postrigor SSP contained more actomyosin and less free myosin than prerigor SSP. Expecting different protein-protein aggregations to occur for pre- and postrigor SSP upon heating would be reasonable. The failure to see such difference in our study is difficult to explain, but actomyosin may have begun to dissociate before a transition temperature for protein-protein interaction was reached. Dissociation of the actomyosin complex in 0.6 M KCl into actin and myosin has been shown to take place at temperatures near 45°C (Jacobson and Henderson, 1973). Thus, differences in protein-protein interaction between actomyosin and myosin may diminish at 45°C or higher.

CONCLUSIONS

THERMALLY INDUCED denaturation and protein-protein interaction of SSP were greatly dependent on pH. Stability of SSP decreased with reduction in pH, showing a decrease in Tm. No pronounced difference in conformational change or denaturation between red and white, and between pre- and postrigor SSP, was detected. Nevertheless, a significant difference in rate and number of thermal transitions of protein-protein interaction was seen between red and white SSP. Thus, we suggest that the conformational change for individual SSP molecules, whether from red or white, or from pre- or postrigor muscle, follows a similar mechanism. However, interaction and association of SSP molecules vary, depending on muscle type. Through protein molecular interaction, textural properties of meat products are conveyed. Thus, the results in this study may partially explain why processed meats at different pH values, and/or when different types of muscle are used, vary in quality.

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Physicochemical and Gelation Properties of Pre- and Postrigor Chicken Salt-soluble Proteins

Y.L. XIONG and C.J. BREKKE

ABSTRACT

The physicochemical and gelation properties of salt-soluble proteins (SSP) extracted from chicken muscles were studied at 0.6M NaCl, pH 6.00. Thermally induced protein unfolding and protein-protein interaction were determined by 8-anilino-1-naphthalene sulfonate (ANS) fluorescence and turbidity. Breast and leg SSP showed similar changes in protein unfolding, but differed in protein-protein interactions. Postrigor breast SSP formed stronger and more elastic gels than prerigor breast and pre- and postrigor leg SSP. Leg SSP gelation was less affected by muscle rigor state than breast SSP. Protein conformational changes were concluded to precede SSP association, which was a prerequisite for gel formation.

INTRODUCTION

AS GENERALLY ACCEPTED, gelation of muscle proteins is largely responsible for binding properties at the junction between meat pieces in comminuted, restructured and formed products. Muscle protein gelation requires that myofibrillar proteins be soluble (or partially soluble) and dispersed in the brine phase of the formulation. The importance of protein solubilization in gelation, as well as in gelation-related properties such as binding, has been elucidated through fundamental studies and in work directly relevant to meat processing (Fukazawa et al., 1961; MacFarlane et al., 1977; Samejima et al., 1985; Foegeding, 1987; Xiong and Brekke, 1989).

The extracted salt-soluble protein (SSP) is ordinarily a mixture of various kinds of proteins, such as myosin, actomyosin and other minor structural and regulatory myofibrillar components. The mechanisms of thermally induced gel formation of individual proteins, such as myosin, actin and actomyosin, have been extensively investigated (Yasui et al., 1982; Asghar et al., 1984; Morita et al., 1987; Samejima et al., 1988; Yamamoto et al., 1988). In general, these studies have shown that myosin by itself forms excellent gels, and actin has poor gelation properties but has a synergistic or antagonistic effect on myosin gelation, depending on the myosin/actin ratio.

The gelation mechanism of SSP is less understood. Foegeding (1987) reported postrigor turkey breast SSP had more favorable gelation properties (shear stress and strain at failure and gel hardness) than thigh SSP, and the gel properties were dictated by pH and ionic strength. Gelation properties of chicken SSP, however, have not been investigated. Since chickens differ from turkeys (species), some differences in SSP gelation may exist. In addition, SSP extracted from prerigor muscle is different from SSP extracted from postrigor muscle in protein composition (Sayre, 1968). For example, the salt extract of chicken pectoralis muscle contains mainly free myosin, with little actomyosin prior to 3 hr postmortem (Sayre, 1968). Hence, some differences between pre- and postrigor muscles in SSP gelation can be expected.

The molecular events involved in the process of protein gelation have drawn the attention of a number of protein chemists (Hermansson, 1978; Acton et al., 1981; Ziegler and Acton, 1984; Foegeding et al., 1986; Foegeding, 1988). Hermansson (1978) suggested protein denaturation prior to aggregation resulted in a finer gel structure, exhibiting a greater elasticity than if random aggregation occurred simultaneously or prior to denaturation. Foegeding et al. (1986) showed heating rate had a great effect on protein-protein association and properties of the resultant myosin gel. It was suggested that the variation in myosin gelation properties could be the result of association of proteins in different denatured states (Foegeding, 1988).

The objective of our study was to determine the effect of pre- and postrigor protein extraction on gelation properties of chicken SSP, and to explore the relationship between thermally induced changes in protein conformation and interaction and gelation properties of these SSP molecules.

MATERIALS & METHODS

Muscle source and preparation

White Leghorn laying hens (17–18 months old) purchased from a local poultry farm were used as the source of muscle for this study. Within each replication, samples were obtained from pooled muscles of two chickens. After 2 min bleeding, the right breast (pectoralis major and minor combined) and leg (thighs and drumsticks combined) muscles were excised and chilled in crushed ice for 5 min. The muscles were then subjected to sample preparation (removal of external fat and heavy connective tissue, cutting and grinding) for salt-soluble protein (SSP) preparation. The total time elapsed between death and the beginning of myofibril isolation was less than 25 min.

The unboned left breast and leg were used as the source of postrigor muscle. The breast and legs, with skin removed but bones attached, were excised from the carcass immediately after bleeding, and chilled in crushed ice for about 30 min. They were then wrapped in paper towels moistened with 1 mM sodium azide (NaN_3), packed in 3 mil polyethylene plastic bags, and stored in an ice slurry for 24 hr before use.

Salt-soluble protein (SSP) preparation

Myofibrils (free of sarcoplasmic and connective tissue proteins) were isolated from the muscle samples, and SSP was prepared from the myofibrils using the procedure described previously (Xiong and Brekke, 1989).

Concentration of protein was determined by the biuret method (Gornall et al., 1949) using bovine serum albumin as the protein standard.

Fluorescence determination

SSP was suspended (0.1 mg/mL protein) in 0.6 M NaCl, 50 mM piperazine-N,N bis (2-ethane sulfonic acid) (PIPES). 1 mM NaN_3, pH 6.00. The SSP suspensions were heated at 1°C/min from 18 to 80°C in a water bath. The sample temperature was constantly monitored with a thermometer. Samples, after reaching specific temperatures, were removed from the bath, cooled and stored in an ice slurry for not more than 3 hr before analysis.

Thermally induced protein conformational change (structure unfolding) was determined essentially according to the method developed by Li-Chan et al. (1985) using 8-anilino-1-naphthalene sulfonate (ANS) as the fluorescence probe. Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer (J4-8961) (American Instrument Co., Division of Travenol Lab., Inc., Silver Spring, MD). Ten microliters of ANS (8.0 mM in 50 mM PIPES, pH 6.0) were added to 2-mL protein solutions. The relative fluorescence intensity

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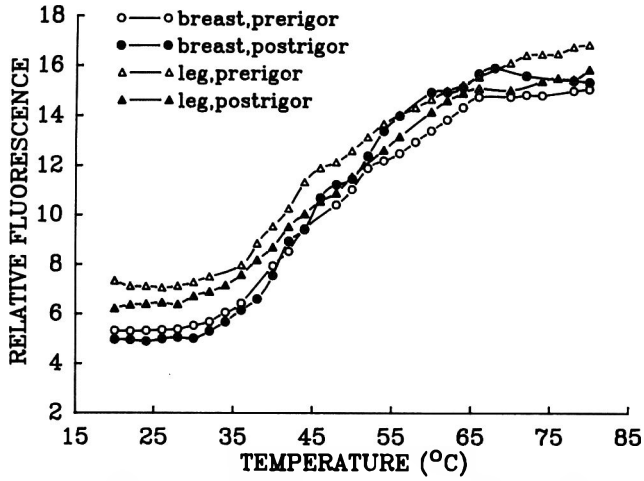

Fig. 1—ANS-protein fluorescence intensity of salt-soluble proteins (SSP) prepared from pre- and postrigor chicken muscles as a function of temperature. Data points represent means from three replicates. SSP was suspended (0.1 mg/mL) in 0.6 M NaCl, 50 mM PIPES, 1 mM NaN_3, pH 6.00; heating rate = 1°C/min.

(RFI) read from the fluorometer was measured 45 min after ANS addition. ANS-protein conjugates were excited at 390 nm, and the fluorescence was determined at emission wavelength 468 nm; the excitation and emission slits were 1 and 2 nm, respectively. "Protein blank" fluorescence values were measured in the absence of ANS and were subtracted from the sample (with ANS added) fluorescence values. The RFI was used to represent hydrophobicity (unfolding) of the protein samples as suggested by Wicker et al. (1986).

Turbidity measurement

Thermally induced protein-protein association/interaction was determined by monitoring the turbidity change of the protein suspension during heating, using the procedure described by Acton and Dick (1986). SSP was suspended (0.3 mg/mL protein) in 0.6M NaCl, 50 mM PIPES, 1 mM NaN_3, pH 6.00. The protein solution was continuously heated at 1°C/min from 18 to 75°C in a temperature-controlled water jacket which was part of a Perkin-Elmer Lambda 5 UV/VIS Spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) a 3mL sample (0°C) was placed in a quartz cuvette and allowed to equilibrate 5 min at the initial temperature of the heating bath (18°C). Sample turbidity changed little during this tempering process, indicating a negligible protein-protein aggregation. Increasing turbidity of the protein solution due to protein-protein association was recorded every minute by measuring changes in optical density at 320 nm. Temperature of samples was monitored with a thermocouple.

Gel preparation and gel property tests

SSP gels were prepared and gel strength (penetration force) measured as previously described (Xiong and Brekke, 1989). Gels were made by heating at a constant heating rate of 1°C/min from 18 to 80°C in a water bath and stored 12 hr at 0°C before testing.

Gel elasticity was determined from the force-penetration curve obtained from the gel strength measurement as described by Hickson et al. (1982).

The gels, after back-extrusion, were centrifuged at 10,000 × *g* for 15 min to study water holding ability (WHA). The centrifuge tube was inverted, and the liquid layer from centrifugation was collected. The expelled liquid (supernatant) and the remaining "gel" (pellet) were weighed. WHA of the gels was determined as moisture loss (g) after centrifugation divided by initial gel weight (g).

Replications

In this study, data of specific parameters (e.g., gel strength and elasticity) were mean values from three replications. Replications refer to experimental work from different protein (SSP) preparations on different days. For each protein preparation, two chickens were used. In each replication, duplicate or triplicate samples were prepared and tested. Difference between means was determined by the Student's t-test (Steel and Torrie, 1960).

RESULTS & DISCUSSION

Hydrophobicity

Changes in hydrophobicity (ANS fluorescence) of the SSP as a function of heating temperature (1°C/min) are shown in Fig. 1. The changes can be divided into approximately three temperature zones. At temperatures less than 35°C, only minor changes in hydrophobicity were observed. At temperatures between 35 and 65°C, hydrophobicity increased rapidly and reached a plateau upon further heating above 65°C. The most dramatic change occurred between 35 and 45°C, suggesting rapid unfolding of proteins within this range. In general, hydrophobicity increased threefold upon heating the samples to 80°C for all SSP samples. Similar results were reported by Li-Chan et al. (1985) for salt-extracted beef protein. Since SSP was essentially a mixture of all myofibrillar components, the observed broad temperature range for change in fluorescence due to protein unfolding was expected. Furthermore, the wide temperature range may also be a result of unfolding of different domains of myosin which require different temperatures. This would be substantiated by the fact that myosin normally shows several thermal transitions at different temperatures (Wright and Wilding, 1984).

Although leg SSP appeared to have greater hydrophobicity than breast SSP at temperatures less than 40°C, leg and breast SSP samples generally showed similar changes in hydrophobicity during heating. This was consistent with the observation of Dudziak et al. (1988) for turkey red and white myosins. Moreover, there was no apparent difference between pre- and postrigor samples. Hydrophobicity change upon heating, as determined by ANS fluorescence probe, is a measure of the extent of exposing hydrophobic regions (Li-Chan et al., 1985; Nakai and Li-Chan, 1988). Hence, the rate and extent of protein unfolding or conformational change appeared similar for breast and leg, and for pre- and postrigor SSP.

Ziegler and Acton (1984) reviewed and summarized conformational changes during thermal denaturation of natural actomyosin. In the 30-35°C range, native tropomyosin is dissociated from the F-actin backbone, while at 38°C the F-actin superhelix dissociates into single chains. The myosin light chain subunits dissociate from the heavy chains at about 40°C, followed by conformational changes in the head and hinge regions of the myosin molecules. Dissociation of the actin-myosin complex occurs at 45–50°C, and light meromyosin undergoes a helix-to-coil transformation at 50–55°C. Above 70°C, major conformational changes in G-actin take place. Note, however, that the exact temperatures for these changes can be influenced by such factors as ionic strength, pH, heating rate, muscle source and type (Stabursvik and Martens, 1980; Wright and Wilding, 1984). Thus, in our study at temperatures below 35°C the small increase in fluorescence intensity (Fig. 1) may correspond to changes in the tertiary structure of F-actin and dissociation of some myofibrillar proteins not yet understood. The rapid increase in fluorescence intensity above 40°C can be ascribed to conformational changes related to myosin denaturation.

Turbidity

Thermally induced protein-protein interaction/association of SSP samples is shown in Fig. 2. Turbidity (increasing light scattering or optical density) increased rapidly when the SSP was heated to about 40°C, indicating the commencement of heat-induced protein-protein interaction or aggregation around 40°C. The rate of the interaction appeared to fall within three temperature zones, namely, <40°C, 40–45°C and >45°C (Fig. 2). Below 40°C, there was little change in optical density, thus little protein-protein interaction. At temperatures between 40

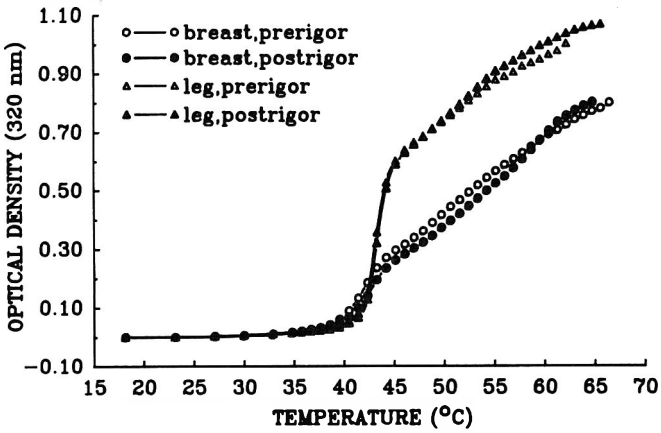

Fig. 2—Protein-protein interaction (turbidity change) at pH 6.00 for salt-soluble proteins extracted from pre- and postrigor chicken breast and leg muscle tissues. Assay conditions: 0.3 mg/mL protein, 0.6M NaCl, 50 mM PIPES, 1 mM NaN_3, pH 6.00; heating rate = 1°C/min.

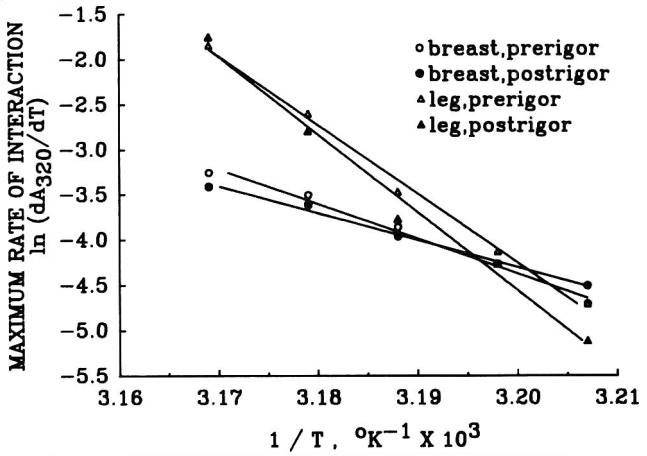

Fig. 3—Arrhenius plots of thermally induced protein-protein interaction at 39–44°C for salt-soluble proteins extracted from pre- and postrigor chicken breast and leg muscle tissues. Data points represent means of five replicates. The differential change in optical density as a function of temperature (dA_{320}/dT) was calculated from the turbidity curve in Fig. 2.

Table 1—Apparent activation energy (E_a) for thermally induced protein-protein aggregation of salt-soluble proteins (calculated from Fig. 3)

	Temperature range (°C)	E_a (J/mol)
Breast, prerigor	39–44	319 ± 27[a]
Breast, postrigor	39–44	244 ± 37[b]
Leg, prerigor	39–44	626 ± 63[c]
Leg, postrigor	39–44	718 ± 67[c]

[a,b,c] Means with different superscripts are significantly different (P < 0.05).

and 45°C, a maximum rate of change was clearly seen. Changes above 45°C were steady but at a reduced rate.

Prerigor and postrigor SSP appeared to follow similar mechanisms of protein-protein interaction, probably because the actomyosin complex in postrigor SSP sample became dissociated around 40°C (Jacobson and Henderson, 1973), where rapid protein-protein interactions were initiated. However, breast SSP showed a great difference from leg SSP (Fig. 2). The onset temperature (when a change in O.D. of 0.02 from initial values occurred) for the protein-protein aggregation was less for breast than for leg proteins. Furthermore, protein-protein aggregation was much more rapid for leg than for breast SSP at 40–45°C. This discrepancy may be related to the isozymic forms of myofibrillar proteins (e.g., myosin) between red and white muscles (Asghar et al., 1985; Morita et al., 1987).

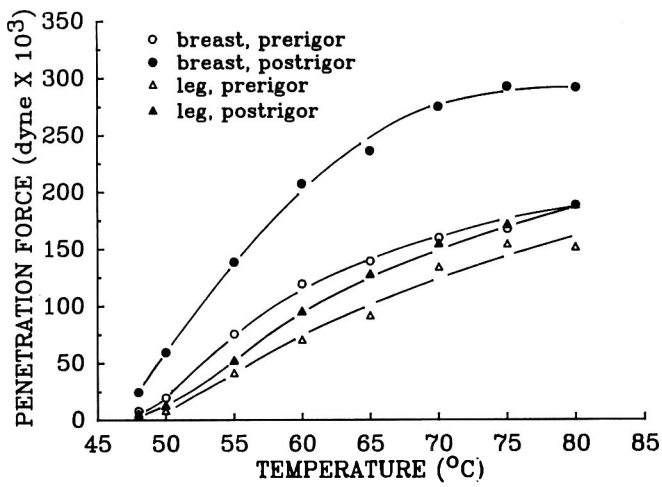

Fig. 4—Effect of heating temperature on gel strength of salt-soluble proteins prepared from pre- and postrigor chicken muscles (mean of three replicates). Gelation conditions: 20 mg/mL protein in 0.6M NaCl, 50 mM PIPES, 1 mM NaN_3, pH 6.00; heating rate = 1°C/min.

Arrhenius plots of the SSP interaction at maximum rate of aggregation (39–44°C) are depicted in Fig. 3. Two different reaction mechanisms, manifested by the different slopes, were evident for breast and leg SSP. Yet, both reactions apparently followed first-order kinetics. Similar behavior has also been observed for bovine actomyosin (Acton et al., 1981) and myofibrils (Samejima et al., 1985) with protein-protein aggregation. The activation energy (E_a) derived from the slopes of the Arrhenius plots is given in Table 1. Leg SSP required more ($P < 0.05$) heat input and was more temperature-dependent than breast SSP during thermal aggregation. Moreover, energy requirement was different ($P < 0.05$) between prerigor and postrigor breast SSP, although for leg SSP samples such difference was not significant ($P > 0.05$).

Gel properties

In general, gel strength increased with increase in temperature, showing a rapid enhancement during the initial stage of gelation (50–60°C) (Fig. 4). The SSP suspension remained fluid and paste-like, and no gel formation was detected at less than 48°C for any SSP samples studied. Postrigor breast SSP formed stronger ($P < 0.05$) gels that did prerigor breast and leg SSP. The gel strength of prerigor breast SSP varied considerably between replications, but, in most cases, prerigor breast SSP produced more rigid gels ($P < 0.05$) than leg SSP. No significant differences were seen between pre- and postrigor leg SSP . The ability of breast SSP to form stronger gels than leg SSP was in good agreement with observations elsewhere that poultry myofibrillar proteins from white muscle form stronger gels than those from red muscle (Asghar et al., 1984; Foegeding, 1987; Morita et al., 1987; Dudziak et al., 1988). The difference in strength between white and red SSP gels could, at least, be attributed to the polymorphism of myosin molecules. Morita et al. (1987) have shown evidence that chicken breast (white) myosin molecules in salt solution associate to form longer and somewhat more ordered filaments than leg myosin molecules. Longer myosin filaments form more rigid gels than short myosin filaments (Yamamoto et al., 1988).

The apparent elasticity of SSP gels is shown in Fig. 5. Although the general pattern of the temperature-dependence of gel elasticity was similar to that for gel strength (Fig. 4), the data points on gel elasticity were more scattered. Hickson et al. (1982) found inconsistent elasticity data with bovine plasma

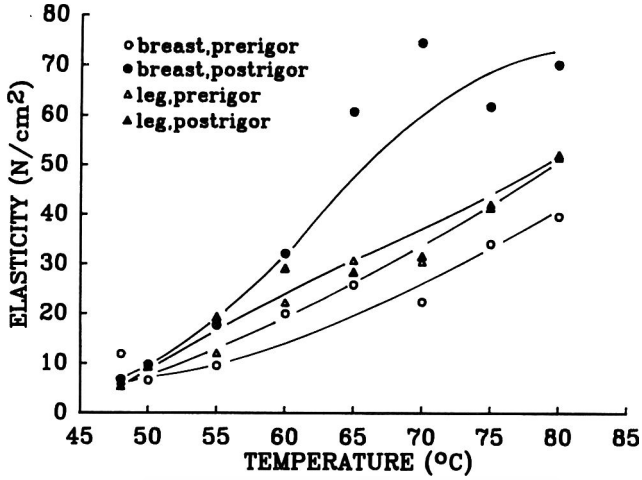

Fig 5—Apparent elasticity of salt-soluble protein gels of pre- and postrigor chicken muscles. Data points represent means of three replicates.

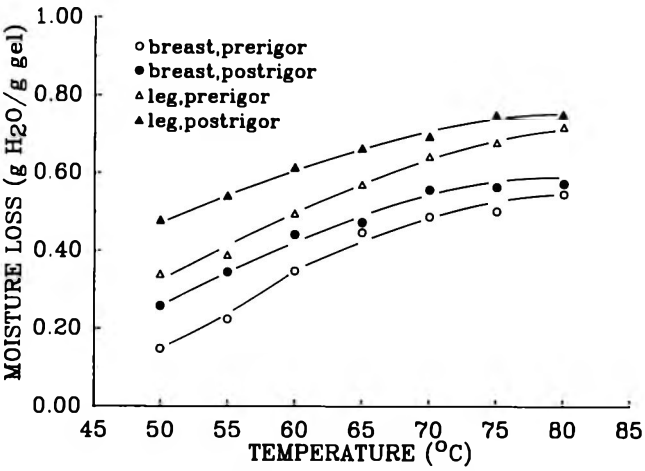

Fig 6—Moisture loss of the pre- and postrigor salt-soluble protein gels by centrifugation as a function of temperature. Data points represent means of three replicates.

Table 2—Approximate onset temperature (°C) for thermally induced protein unfolding, protein-protein interaction and gel formation of salt-soluble proteins (pH 6.00)

	Protein unfolding (ANS-fluorescence)	Aggregation (turbidity)	Gelation (Instron)
Breast, prerigor	30	36	48
Breast, postrigor	32	36	48
Leg, prerigor	30	39	50
Leg, postrigor	30	40	48

gels. In spite of the apparently great elasticity of postrigor breast gels, prerigor breast SSP gels appeared similar to leg SSP gels. Elasticity represents a different gel property than gel strength, thus the two would not necessarily correlate.

The different gelling properties observed between prerigor and postrigor SSP (moreso for breast than for leg SSP) can be explained by the different compositions of the SSP. In prerigor samples, the salt extract presumably consisted mainly of myosin and actin unassociated. In postrigor salt extract, however, the principal protein should be actomyosin. Sayre (1968) reported the ratio of myosin to actomyosin (w/w), extracted from chicken pectoralis in 0.76M KCl, 0.1M phosphate, pH 7.0, decreased from 7 (0.5 hr postmortem) to 0.8 after 24 hr postmortem. Myosin and actomyosin have quite different gelation behaviors (Yasui et al., 1982; Morita et al., 1987; Samejima et al., 1988). The gel strength of a mixture of myosin and actomyosin varies according to the ratio (w/w) of myosin/actomyosin with maximum often observed at a ratio of about 4. In our study, SSP in the postrigor salt extract may have a myosin/actomyosin ratio that favored stronger gel formation than SSP in the prerigor salt extract.

WHA of the gels decreased with increased heating, which was indicated by the increasing amounts of expressed liquid as a result of higher heating temperature (Fig. 6). This is because heating induced protein denaturation and conformational changes that increased the exposure of hydrophobic regions (Fig. 1) and resulted in aggregation of proteins (Fig. 2). As a consequence, the interfilamental space of proteins in the gel was condensed so that less water could be physically entrapped in the space. In addition, the hydration capacity of protein or protein-solvent (water) interaction was decreased after the protein was denatured. Similar results were reported by Hermansson and Lucisano (1982) for blood plasma gels.

Differences between leg and breast, and between pre- and postrigor SSP, were apparent ($P < 0.05$) (Fig. 6). The leg SSP gels showed greater moisture loss after centrifugation than did the breast SSP gels. Prerigor SSP, of either leg or breast, retained more water in the gels than postrigor SSP, although as shown previously, prerigor SSP gels were more easily ruptured than postrigor SSP gels (Fig. 4). We suggest that the protein matrix formed during gelation may vary with muscle type and rigor state, which can be ultimately ascribed to the different proteins/ isoforms that contributed to the gel network formation. Hermansson (1982) substantiated that the water binding property of blood plasma protein gel is closely related to the microstructure of the gel matrix. Gel microstructure possibly differed for breast and leg, and for pre- and postrigor SSP, which caused variations in moisture loss from one type of gel to another, as observed in our study.

Ferry (1948) proposed gelation of protein proceeds by a two-step mechanism of denaturation and association. Foegeding (1988) further developed Ferry's theory by elaborating the molecular event occurring between protein denaturation and association. He indicated gel properties of large molecules (e.g., myosin) can be dictated by the way proteins associate. The association of protein molecules can be influenced by different domains of the molecules, and the rate of unfolding within domains can depend on heating conditions.

In our study, the thermally induced chicken SSP protein unfolding (ANS fluorescence) and association (turbidity), as well as gelation, were conducted at constant heating rate (1°C/min). The onset temperatures at which the initial change in protein denaturation or gelation occurred are summarized in Table 2. Despite some differences between breast and leg, and between prerigor and postrigor SSP samples, a general trend was seen in all samples that protein-protein association/interaction (beginning at 36–40°C) was preceded by protein unfolding (30–32°C). Gelation (45–50°C) was initiated after interaction of proteins had taken place (Table 2). There were some similarities between our results and reports from other studies on myosin (Foegeding et al., 1986; Wicker et al., 1986). Foegeding et al. (1986) showed association of porcine leg myosin molecules can be detected prior to gelation at heating rates of 0.2°C/min or 0.8°C/min. Wicker et al. (1986) reported thermal transition in protein unfolding (ANS fluorescence) preceded the gel rigidity transition of myosins prepared from chicken breast, rabbit and tilapia (fish) upon heating at 1°C/min.

It must be emphasized, based on our results, that chicken SSP gelation under the conditions we used (0.6M NaCl, pH 6.00, heating rate of 1°C/min) follows the sequential changes of protein unfolding → protein-protein association → gelation. However, the degree of protein unfolding prior to aggregation, and the extent of protein aggregation before forming a gel matrix, remain unknown. Gelation might possibly start to take place after proteins are partially denatured, and both denatured and partially denatured proteins would seem to participate in

molecular interaction and aggregation in forming the protein-protein network. This is speculated because thermally induced changes in protein unfolding (Fig. 1), interaction (Fig. 2) and gelation (Fig. 4) all occurred over a wide temperature range, temperature overlapped considerably where these different molecular changes/interactions took place.

CONCLUSIONS

THERMAL DENATURATION of protein and subsequent protein-protein association proved to be necessary before an elastic and rigid SSP gel could be formed. Therefore, in meat processing, the thermal treatment, including the heating rate, must be carefully controlled to allow sufficient time for protein to unfold and aggregate prior to gel formation. Furthermore, moisture retention of the SSP gels decreased with increasing temperatures, although gel strength was directly proportional to temperature. These two opposite heating effects must be balanced in thermal processing so that a well-bound and textured, as well as tender and juicy, meat product can be produced. In addition, the different gelation properties of pre- and postrigor and of breast and leg SSP need to be considered in formulation of meat products.

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Effect of NaCl and KCl on Rancidity of Dark Turkey Meat Heated by Microwave

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ABSTRACT

Patties from six different formulations containing ground dark turkey meat, water, sodium chloride, and/or potassium chloride were cooked in a microwave oven and stored (4°C) for nine to thirteen days with multiple reheating of the same patties. Thiobarbituric acid analyses revealed that multiple reheating retarded lipid oxidation, including that caused by NaCl (2.0%) which was a pro-oxidant compared to KCl (2.0%). Sensory panelists rated formulations containing ≥ 1.0% KCl more rancid than any others except that containing no added salts. Panelists found no statistical differences in rancidity of patties related to number of times reheated.

INTRODUCTION

LIPID OXIDATION causes deterioration of meat quality, especially during heating and subsequent storage. Warmed-over flavor (WOF), the rapid onset of lipid oxidation in cooked, refrigerated meat, can be detected within 48 hr after heating. Oxidized flavor, characteristic of rancid meat, develops over a longer period of time, usually during frozen storage (Pearson and Gray, 1983).

Grinding and exposure to air can hasten onset of rancidity in ground turkey muscle (Greene, 1969; Sato and Hegarty, 1971). Thus, WOF is a serious problem in reformed products made from ground dark turkey muscle which has a low α-tocopherol concentration and an abundance of unsaturated fatty acid in the phospholipid fraction of fat (Dawson and Gartner, 1983). Wilson et al. (1976) ranked the susceptibility of several different types of meat to WOF as: turkey > chicken > pork > beef > mutton.

NaCl, added to several meat products for its functional properties, was shown to promote lipid oxidation in dark poultry meat (Dawson et al., 1978; King and Earl, 1988). King and Earl (1988) reported a 50% substitution of NaCl (1.0%) with KCl significantly reduced thiobarbituric acid (TBA) values in turkey patties that were frozen before cooking. Apgar and Hultin (1982) observed an increased rate of enzymic peroxidation in fish muscle microsomes (−12°) in presence of NaCl, compared to KCl. One report suggested that metal impurities in NaCl, and not some effect of the salt, increased lipid oxidation (Salih, 1986). Other investigators reported that cations from salts were pro-oxidants (Castell et al., 1965).

To keep pace with consumer demand for convenience items, several food processors now produce preheated or ready-to-serve poultry products (containing added NaCl) that can be heated in the microwave. While WOF and rancidity are detectable in some products heated by microwave, the degree of lipid deterioration is less than that caused by conventional heating methods (Steiner et al., 1985). That finding suggested that heating or reheating by microwave could retard the pro-oxidizing effects of NaCl. The purpose of our research was to evaluate the effect of exhaustive microwave heating on rancidity of ground, dark turkey meat containing added NaCl and KCl.

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MATERIALS & METHODS

Additives

NaCl, KCl, and Lite Salt (a 50:50 blend of NaCl and KCl) were donated by Morton Salt (Division of Morton Thiokol, Inc., Chicago, IL).

Iron, copper, and manganese content of additives

Iron, copper, and manganese content of NaCl, KCl, and Lite Salt were determined by inductively coupled plasma spectrometry (ICP) at the Division of Agriculture and Natural Resources Diagnostic Laboratory at the University of California, Davis, CA. Six grams of each salt were dissolved in 25 mL of triple distilled water; the solution was pumped into the nebulizer of the spectrometer at a rate of 2 mL/min.

Mixing of formulations

Refrigerated, deboned thigh meat from 19-week-old male turkeys was obtained from a local processor. The turkeys were slaughtered approximately 5 hr prior to delivery of meat to the laboratory. Removal of skin and adhering fat, grinding, mixing, and storage of meat were conducted in a cold room (4°C). Meat (74.7% moisture, 6.8% fat) was always stored in closed plastic containers.

One kg batches of meat were chopped with the cutting blade of a food processor (Model DLC 7, Cuisinarts, Norwich, CT) for 30 sec. Ground meat (4.5 kg) was premixed in a mixer (Model 120 T, Hobart Corp., Troy, OH) for 3.0 min at speed 1. A hand-held aluminum utensil was used to thoroughly mix all ground, premixed meat into one large composite. Meat was then divided into smaller batches; salt (w/w) and deionized distilled water (10% v/w) were added separately. The combinations and percentages of salts added were: 0%, NaCl (2.0%), KCl (2.0%), NaCl (1.0%) + KCl (1.0%), NaCl (1.5%) + KCl (0.5%), or NaCl (0.5%) + KCl (1.5%). The latter three combinations contained Lite Salt alone or combined with NaCl or KCl. Each formulation was thoroughly mixed 5 min in the mixer at speed 1, then stored (4°C) an additional 15–18 hr. Patties were formed according to procedures of King and Earl (1988) and stored for about 15 hr before cooking. Excess meat not used to make formulations was frozen (−20°C) 5 months and used in Study II.

Study I: Objective measurement

Cooking and reheating of patties. Eighteen patties per formulation were cooked in a microwave oven (Model 1310-S, 12 volts, Hobart Corp., Troy, OH). During each 12 min heating period, eight patties (at least one from each formulation) were heated at maximum power; they were turned to alternate sides and moved to a new position in the oven every 3 min to obtain a total cooking time of 6 min on each side. Patties were kept in the oven for 1 min without power before they were removed. After heating 6 min and holding 1 min, there was no visible red or pink color on the surface of patties; the internal temperature of patties after the final 1 min holding time was 78°C. After draining 5.0 min on each side, cooked patties were stored (4.0°C) without drained liquid in doubled reclosable plastic bags inside plastic containers 1 to 13 days.

Defining the first day of cooking as day 1, all patties from each formulation were reheated on days 2, 5, 7, 9, 12, and 14. Thus, patties analyzed for TBA values (see below) on day 2 had been reheated once while those analyzed on day 14 had been reheated six times. Patties were reheated inside the plastic bags in the microwave oven for 40

Table 1—Effect of formulation on TBA values of cooked dark turkey meat

Formulation	TBA values[z]
Meat	8.1 ± 2.0[b]
NaCl (2.0%)	9.1 ± 2.0[a]
KCl (2.0%)	8.1 ± 2.4[b]
NaCl (1.0%) + KCl (1.0%)	8.4 ± 1.9[b]
NaCl (1.5%) + KCl (0.5%)	8.7 ± 2.1[ab]
NaCl (0.5%) + KCl (1.5%)	8.1 ± 2.5[b]

[z] TBA value = mg malonadehyde/kg meat. N = 30. Means within column with different superscripts differ significantly at P<0.05.

Table 2—Selected metal content of additives added to dark turkey meat

	Total metal content (ppm)[z]		
Salt	Iron	Manganese	Copper
NaCl	<0.03[a]	<0.01[a]	<0.05[a]
KCl	<0.03[a]	<0.01[a]	<0.05[a]
Lite salt[y]	0.15[b]	0.02[b]	0.24[b]

[z] N = 6, means within column with different superscripts differ significantly at P<0.05.

[y]Lite salt is a 50:50 blend of NaCl and KCl.

Table 3—Effect of reheating on TBA values of cooked dark turkey meat

	TBA values[z]				
	Day of reheating				
Treatment	2	7	9	12	14
Control	5.3[ab]	7.0[b]	8.6[b]	8.9[c]	10.7[a]
NaCl (2.0%)	5.9[a]	8.4[a]	10.0[a]	10.5[a]	10.9[a]
KCl (2.0%)	4.4[bc]	7.2[b]	9.1[ab]	9.6[bc]	10.4[a]
NaCl (1.0%) + KCl (1.0%)	5.4[a]	8.1[a]	8.8[b]	9.6[bc]	10.3[a]
NaCl (1.5%) + KCl (0.5%)	5.6[a]	7.7[ab]	8.9[ab]	9.9[ab]	11.2[a]
NaCl (0.5%) + KCl (1.5%)	4.2[c]	7.7[ab]	8.3[b]	9.4[bc]	10.9[a]

[z] TBA value = mg malonadehyde/Kg meat. N = 6, means within columns with different superscripts differ significantly at P<0.05.

Table 4—Effect of formulation, reheating, and storage on TBA values of cooked dark turkey meat

Formulation	TBA values[z]	
	Reheated	Stored
Meat	15.2 ± 1.1[c]	18.5 ± 0.5[a]
KCl (2.0%)	10.1 ± 2.0[e]	15.1 ± 1.0[c]
NaCl (2.0%)	13.1 ± 0.5[d]	16.5 ± 0.4[b]

[z] TBA value = mg malonadehyde/kg meat. N = 12, means with different superscripts differ significantly at P<0.05.

sec at maximum power. The internal temperature of patties after reheating was 85.5°C.

TBA values. The method of Salih et al. (1987) was used to determine TBA values (mg of malonaldehyde/Kg of meat) for all patties as follows. Three of the remaining patties from each formulation were analyzed and discarded after reheating on day 2 and after each subsequent reheating (days 5, 7, 9, 12, and 14). Thus, only intact patties were used for each TBA analysis. Unfortunately, reliable data for day 5 was collected for the second run only and could not be included in the statistical analysis.

Study II: Effect of storage and reheating

Previously frozen skinned and deboned meat from Study I was thawed and prepared as three formulations with added water (10% v/w) and salts [(0%, NaCl (2.0%), or KCl (2.0%)]. Twenty-four patties from each formulation were cooked and stored as reported in Study I. Twelve patties from each formulation were reheated on days 2, 5, 7, 9, 12, and 14 by the reheating procedure mentioned in Study I. On day 14, TBA values of all patties were determined.

Study III: Sensory evaluation

Evaluation 1. Meat was received, made into formulations, cooked, and stored as described in Study I. Defining day of cooking as day 1, each patty was reheated on days 2, 5, 7, and 9 for 40 sec at maximum power; they were evaluated on day 9. Wedges of approximately equal size were cut from patties and put into 80 mL paper cups, previously placed inside closed styrofoam containers. Sample wedges were warmed inside closed containers in a microwave oven 15 sec at 80% maximum power. They were immediately served to eight sensory panelists, members of the Department of Avian Sciences at the University of California, Davis, CA. Training of panelists was conducted according to Guidelines for the Selection and Training of Sensory Panel Members (1981). Panelists evaluated sample wedges for degree of rancidity by the deviation-from-reference method under red illumination (Feria-Morales and Pangborn, 1983). A non numerical 10-cm graphic scale was used to evaluate each sample. Two samples per formulation per panelist were compared to a control that was processed and prepared on the day of the evaluation. The control had a pre-set score of 5.

Evaluation 2. Patties from the formulation con aining NaCl (1.5%) + KCl (0.5%) were prepared and reheated four times (4X): days 2, 5, 7, and 9; three times (3X): days 5, 7, and 9; or once (1X): day 9. Panelists compared two samples per formulation to a reference sample processed and prepared on the day of the evaluation.

Statistical analysis

Data from a single run of Study II, duplicate runs of Studies I and III, and triplicate runs of the determination of metals by ICP were analyzed as a randomized complete block, factorial design. MSTAT Anova, Factor and Range subprograms were employed (Power, 1983). The mean square for replication within formulation was used to test the significance of replication (where applicable) and formulation. The residual mean square was used to test the significance of reheating and formulation × reheating for Studies I, II, and III and the significance of additive for the ICP determination. Means for all main effects and the interaction of formulation × heating were ranked using Duncan's new multiple range test at P<0.05.

RESULTS & DISCUSSION

TBA values and effect of metals

The main effect of formulation on TBA values is shown in Table 1. NaCl (2.0%) significantly accelerated oxidative rancidity by TBA test, when compared to four other formulations and along with formulations containing 1.5% NaCl was statistically different from the others. The effect of NaCl has been thought to be due, in part, to metal content, especially iron (Salih, 1986). Results shown in Tables 1 and 2 did not support that proposal. NaCl (2.0%) with trace amounts of iron and copper was a pro-oxidant when compared to KCl (2.0%) which contained the same amounts of those metals. We did not know whether the oxidation states of the iron (or copper) in the additives were different and variously affected lipid oxidation.

It was important to determine if reheating by microwave would retard the effects of NaCl (2.0%). Inspection of TBA values as time increased showed that on day 14, the degree of rancidity (TBA) for all formulations including that containing NaCl (2.0%), was not statistically different (Table 3). The similarity in values could be attributed to inhibition of oxidation by Maillard reaction products (MRP) produced during heating and exhaustive reheating by microwave (Bailey et al., 1987). Results of Study II suggested that MRP may have retarded lipid deterioration in that patties stored at 4°C had significantly higher TBA values as compared to their counterparts that were stored and subjected to multiple reheating (Table 4). However, the results were complicated by the fact that the level of rancidity (TBA) in meat used in Study II was much greater than that in meat used in Study I. The previously frozen, uncooked meat mixture used in Study II had an initial TBA value > 6 while that used in Study I had a value < 1. Another study using meat with a TBA value < 1 should be undertaken.

The results of Study II were unexpected in that NaCl (2.0%) produced TBA values significantly lower than those of meat with no added salts (Table 4). This suggested that the impact of NaCl on lipid deteriorations occurred during the early storage period. The results of Study I partially supported this observation. The overall pro-oxidizing effect of NaCl (2.0%) and the similarity in TBA values of all formulations by day 14

Table 5—Sensory scores and TBA values of cooked, reheated dark turkey meat.

Formulation	Sensory scores[z]	TBA values[z]
Meat	6.1[bc]	8.6[b]
NaCl (2.0%)	5.7[cd]	10.0[a]
KCl (2.0%)	7.1[a]	9.1[ab]
NaCl (1.0%) + KCl (1.0%)	6.7[ab]	8.8[b]
NaCl (1.5%) + KCl (0.5%)	5.8[cd]	8.9[ab]
NaCl (0.5%) + KCl (1.5%)	6.8[ab]	8.3[b]

[z] TBA value = mg malonaldehyde/kg meat. N = 16 for sensory scores, n = 6 for day 9 TBA values from Study 1, means within columns with different superscripts differ significantly at $P<0.05$.

could be related to the impact of NaCl (2.0%) during the early stages of storage (Tables 1 and 3).

Sensory evaluations

Sensory panelists judged rancidity of patties reheated several times during 9 days of storage. Statistical analysis of data revealed a significant effect of formulation (Table 5). Panelists rated patties containing ≥ 1.0% KCl as similar and more rancid than all others except those containing no added salts (Table 5). The trend for the sensory evaluation was not in agreement with that from the TBA analysis of Study I, day 9 (Table 5). Panelists judged samples containing KCl (2.0%) as more rancid than those with NaCl (2.0%) whereas TBA values had indicated both formulations had a similar degree of rancidity.

The formulation containing NaCl (1.5%) + KCl (0.5%) was evaluated after reheating (1X, 3X, and 4X) to determine if degree of rancidity significantly correlated with the number of times reheated. Panelists rated all reheated samples statistically not different and more rancid than a freshly prepared control. Samples reheated 1X, 3X, and 4X received statistically not different ratings of 6.8, 6.4, and 6.9, respectively. Results of Study II indicated patties stored at 4°C after cooking in the microwave had higher TBA values than those reheated several times. Patties used in our evaluation were always stored at 4°C prior to and after reheating. The combination of storage and reheating possibly resulted in similar degrees of rancidity in all patties that was indistinguishable between them.

The results of our study suggested that (1) reheating of dark turkey meat patties by microwave may retard the pro-oxidizing effect of NaCl and (2) the effect of NaCl (2.0%) occurred during the early stages of storage. Panelists rated the formulation containing KCl (2.0%) as more rancid than that containing NaCl (2.0%) but could not detect differences in rancidity of patties of one formulation related to the number of times reheated.

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Hedonic scales

The scores for flavor preference and overall acceptability were greatest when using the highest cooking pressure and slowest cooling rate during the first study. This indicates the sensory panel used for these tests preferred meat with the most severe temperature-time treatment. Due to the small panel size this may not correspond to consumer response.

CONCLUSIONS

NEITHER COOKING PRESSURE nor pressure change rate during vacuum cooling affected chicken breast tenderness. Significant differences in the sensory scores between treatments were more likely to occur when those treatments imposed greatly differing temperature-time effects. Weight loss increased the more severe the temperature-time treatment. Due to the high weight losses associated with vacuum cooling, air chilling was undoubtedly preferable, unless rapid cooling was an overriding requirement.

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Free Amino Acids in Cheddar Cheese: Comparison of Quantitation Methods

L. LEMIEUX, R. PUCHADES, and R.E. SIMARD

ABSTRACT

Two reported methods were compared with a more rapid and accurate flow injection analysis (FIA) technique. A trinitrobenzenesulfonic acid (TNBS) method provided absolute values, was tedious, and the reagent was light-sensitive and required heating. An *o*-phthaldialdehyde (OPA) method was faster and less variable than the TNBS, but reaction time had to be carefully controlled. The results with the OPA correlated well with the TNBS but were overestimated due to reading small peptides and ammonium ions. The FIA method was simple and reliable, yielded values which correlated closely with amino acids and gave information about degree of proteolysis.

INTRODUCTION

THE DETERMINATION of amino acids in cheese is important because of their significant contribution to good cheese flavor. Amino acids are produced during cheese ripening by the proteolytic action of starter bacteria and rennet (Amantea et al., 1986). The extent of this proteolysis has been used to indicate the degree of cheese ripening maturity (Venema et al., 1987; Ney, 1971). Views about the relationship between amino acid levels and cheese flavor are opposing (Kaminogawa et al., 1986; Pettersson and Sjostrom, 1975). Law and Sharpe (1977), considered amino acids to be intermediate products in production of certain aromatic compounds. Measurement of free amino acid levels is therefore useful for investigation of flavor chemistry in cheese (Nishimura and Kato, 1988). The concentration of free amino acid increases with ripening and its significance as a quantitative indicator of proteolytic activity during ripening (Buruiana and Zeydan, 1982) is well known.

Many procedures have been used to detect proteolysis in dairy products. Traditionally, the most widely used method was described by Hull (1947). The method lacks sensitivity and is cumbersome to use with many samples. Cheese ripening and the degree of proteolysis have been measured by reading absorbance of a sodium citrate-hydrochloric acid extract at 270 and 290 nm (Trepman and Chen, 1980). Alternative assay methods have been proposed (Exterkate, 1979; Mills and Thomas, 1978), which, although sensitive, required sophisticated instrumentation. Methods employing reagents that specifically react with α-amino groups, e.g. ninhydrin (Reimerdes and Klostermeyer, 1976), 2,4,6-trinitrobenzenesulfonic acid (Jarrett et al., 1982) and *o*- phthaldialdehyde (Church et al., 1983) provide increased sensitivity. They are direct determinations of all products of proteolysis, and are currently accepted for assessment of protein degradation in cheese.

The trinitrobenzenesulfonic acid (TNBS) method is a spectrometric assay of the chromophore formed by reaction of TNBS with primary amines. The reaction takes place under slightly alkaline conditions and is terminated by lowering the pH. A linear relationship is generally assumed between the color intensity and the concentration of α-amino groups. *Ortho*-phthaldialdehyde (OPA) reacts with amino acids in alkaline medium in presence of a reducing agent such as 2-mercaptoethanol, giving rise to strongly fluorescing compounds. The OPA method has been widely applied to determination of amino acids and proteins (Hare, 1977; Viets et al., 1978), including automated analysis (Roth and Hampai, 1973), because it is more rapid and convenient than the TNBS method (Church et al., 1983).

These methods, while reliable and sensitive, require considerable time and numerous manipulations of samples, making their routine application difficult or impractical. Due to the great number of analyses required to monitor proteolysis in milk or dairy products, sensitive, precise, and rapid methods are needed. These new techniques such as flow-injection analysis (FIA), have been developed for automation of routine determinations (Puchades et al., 1990). FIA is a dynamic process that produces a well-defined and highly reproducible concentration transient at the detector site (Matsumoto et al., 1988). The coupling of immobilized enzyme reactors with FIA provided increased speed, precision and convenience compared to classical enzymatic procedures (Valcarcel and Luque de Castro, 1987), and decreased cost.

The objective of our study was to compare TNBS and OPA methods with a new sensitive and automated FIA technique for rapid determination of free L-amino acids in Cheddar cheese. This new proposed method was shown useful to measure the extent of protein degradation in different samples of Cheddar cheese.

MATERIAL & METHODS

Reagents

L-amino acid oxidase (L-AAO, EC 1.4.3.2 *Crotalus adamanteus* venom, type V), L-leucine, controlled pore glass (CPG 240-200), trinitrobenzenesulfonic acid, o-phthaldialdehyde and 2-mercaptoethanol were purchased form Sigma Chemical Company (St-Louis, MO, USA). All other reagents were commercially available and of analytical grade. All solutions were prepared with water purified by a Milli Q-system (Millipore, Bedford, MA, USA).

Procedure

Cheddar cheese manufacture. Cheddar cheese was made on a commercial scale by the procedure used by Agropur (Granby, Québec, Canada) as previously described (Puchades et al., 1989).

Sampling. All cheeses were sampled at 2, 4, 7 and 9 months and divided into blocks of 800–900g for further analysis.

Preparation of cheese extract. The water-soluble fraction (WSF) was extracted from the Cheddar cheese by the method of Harwalkar and Elliott (1971).

Sample preparation. Almost 30 mg freeze-dried extract was diluted in 5 mL 0.1M phosphate buffer pH 7.5, sonicated and filtered through a 0.45 μm (Millipore) membrane and a Sep-Pak C18 cartridge (Waters Associates, Part No. 51910). This solution was used in all tests.

TNBS method. Except for extraction of the sample, this method was the same as that described by Adler-Nissen (1979). A 0.250 mL aliquot of sample was mixed in a test tube with 2.0 mL phosphate buffer at pH 8.2. Two milliliters 0.1% TNBS were added, the test tube shaken and placed in a water bath at 50 ± 1°C for 60 min. During incubation the test tubes and the water bath were covered with aluminum foil because the blank reaction was accelerated by exposure to light. After 60 min, 4.0 mL 0.10N HCl were added to terminate

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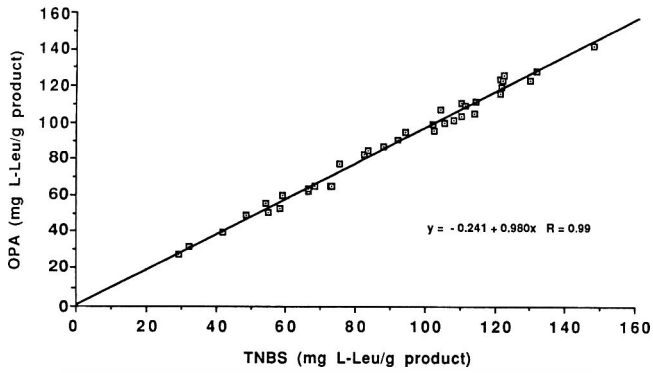

Fig. 1—Relationship between the levels of free amino groups determined by OPA and TNBS methods.

the reaction and the test tube allowed to stand at room temperature (22°C) 30 min before absorbance was read vs. water at 340 nm in a Spectronic 2000 (Bausch and Lomb, NY, USA). The blank reading was subtracted from sample readings.

OPA method. The OPA procedure was a modified version of that described by Church et al. (1983). The OPA reagent was prepared by combining the following reagents and diluting to a final volume of 50.0 mL with water: 25.0 mL of 100 mM sodium tetraborate; 40.0 mg of OPA (dissolved in 1.0 mL of methanol); and 100.0 μL of β-mercaptoethanol. Extracts of cheese (WSF) were used for the reaction with OPA. A small aliquot (150 μL) was added directly to 3.0 mL OPA in a 3.5 mL quartz cuvette; the solution was mixed briefly by inversion and incubated for 2 min at ambient temperature (22°C). The absorbance at 340 nm was measured by a 8451 Diode array (Hewlett Packard, FRG) spectrophotometer.

FIA method. The procedure was described by Puchades et al. (1989b). Using a flow system, 44 μL sample was degraded enzymatically by L-amino acid oxidase immobilized on controlled porosity glass. The generated H_2O_2 was determined by chemiluminescence (CL) with a solution containing luminol (1.5 mM) and hexacyanoferrate (8.0 mM) at pH 10.5. The CL was measured by a Gilson Spectra/glo filter fluorometer. Sample throughput was 40 h^{-1}. The L-leucine standard solution and samples were the same for all methods.

Amino acid analysis was done for three different manufactured Cheddar cheeses and a control, ranging in age from 1–9 months. The amino nitrogen content of 37 cheese samples was determined in triplicate and the results were expressed in mg L-leucine/g product.

RESULTS & DISCUSSION

THE ANALYSIS of cheese of varying sharpness resulted in mean values from the OPA method that were not significantly different ($P < 0.01$) from those when the TNBS method was employed. The OPA values closely correlated (slope = 0.980) with those for amino nitrogen determined by the TNBS method (Fig. 1). TNBS and OPA are frequently cited methods for estimating free amino acids in different products (Hazra et al., 1984; Jarrett et al., 1982; Svedas et al., 1980). However, some difficulties are inherent to each of them. A quantitative reaction between TNBS and the amino groups requires a reaction time of 60 min. When the reaction times for samples and blanks differed by more than a few minutes, a considerable error was introduced because of the blank reaction. This method required heating and all test tubes had to be covered with aluminum foil because the reagent is light-sensitive. This method did not measure proline and hydroxyproline, and was subject to interference from ammoniacal nitrogen (Clegg et al., 1982).

OPA formed adducts of similar high absorptivity (Church et al., 1983) with all but two α-amino groups (a weak reaction with cysteine and none with proline). The interaction between amino acids and OPA was very effective even in dilute solutions at room temperature, and the reaction was usually complete within a few minutes. The recorded optical density of the product solution was directly proportional to the AA concentration (Svedas et al., 1980). The limitation of this method was the delay between addition of the reagent and the reading.

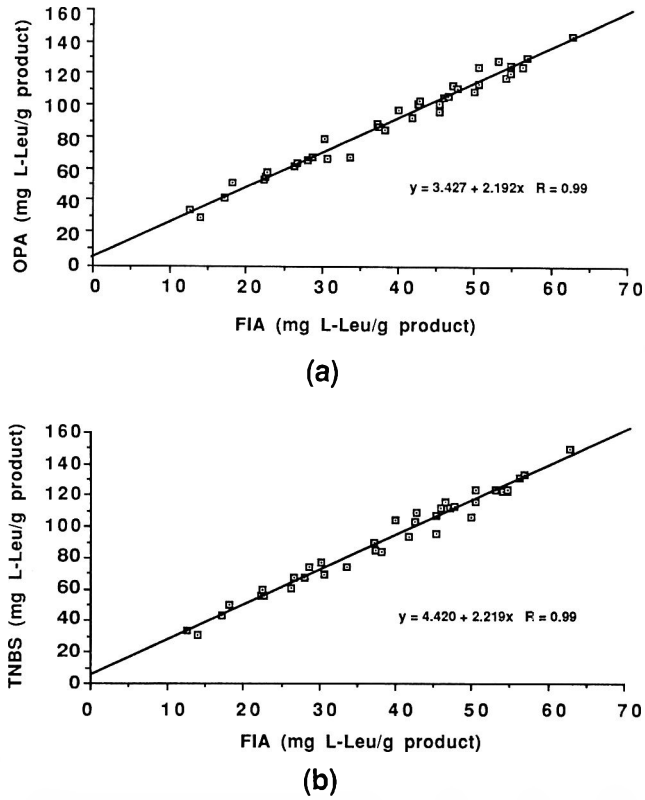

Fig. 2—Relationship between the levels of total free amino acids determined by the (a) OPA and FIA methods; (b) TNBS and FIA methods.

The OPA method was simpler and faster than the TNBS method but it also measured the free amino groups of peptides having a molecular weight smaller than 6000 Daltons.

When free amino acids in the same cheese samples were determined by FIA with an enzymic reactor and chemiluminescence detector, the method was very fast (40 samples h^{-1}), could be run at room temperature (22°C), used simple instrumentation and was very specific. Despite the fact that this method did not measure all amino acids, those specific amino acids which are good indicators of the proteolytic activity in cheese ripening were measured (Nasr and Younis, 1986).

Results of regression and correlation analyses carried out between levels of soluble amino nitrogen by TNBS or OPA methods and free amino acids by FIA; are given in Fig. 2a and 2b. The high correlation ($R = 0.99$) indicated a strong relationship, and the results from the estimations were linearly related.

The reproducibility was tested by analyzing the same sample ten times. The relative standard deviations were lower for the FIA method (0.3%) than for the OPA (2.8%) or TNBS (3.3%) methods. The mean values obtained with both OPA and TNBS methods were higher than those with FIA. Comparison of amino nitrogen measured by TNBS and OPA with amino acid levels, expressed as mg L-leu/g product, measured by FIA gave ratios greater than one. This indicated, in agreement with literature, that some peptide material was reacting with the OPA and TNBS reagents. The FIA method was simpler and more reliable for monitoring free amino acid levels in cheese, and was most effective of the tested methods. Since the concentration of free amino acids in cheese correlates significantly with flavor development and is considered a reliable indicator of ripening, many Cheddar cheeses prepared with different lactobacilli starters (*L. plantarum* 99, *L. casei-casei* 119, *L. casei-casei* L2A, and control) were analyzed by these methods at different stages of ripening (2, 4, 7, and 9 months). Results are given in Table 1.

Table 1—Amino nitrogen levels in Cheddar cheeses measured by TNBS and OPA methods with levels of free amino acids determined by the FIA method

Cheese age (months)	Cheese 1[a]			Cheese 2			Cheese 3			Cheese 4		
	TNBS[b,c]	OPA	FIA	TNBS	OPA	FIA	TNBS	OPA	FIA	TNBS	OPA	FIA
2	114.19	105.30	46.63	32.33	32.27	12.53	59.25	60.45	26.29	29.19	27.62	14.07
4	68.33	65.82	30.62	41.82	40.16	17.24	66.61	62.93	26.74	48.83	49.67	18.16
7	102.26	100.00	42.63	55.03	51.53	22.31	72.75	65.96	33.64	54.41	56.55	22.68
9	110.56	104.41	45.90	58.30	53.62	22.51	88.23	87.70	37.23	66.62	64.32	28.02

[a] Cheeses with added lactobacilli: 1: *L. plantarum* 99; 2: *L. casei-casei* 119; 3: *L. casei-casei* L2A; 4: control.
[b] Results expressed as mg L-leu/g product.
[c] Averages from triplicates.

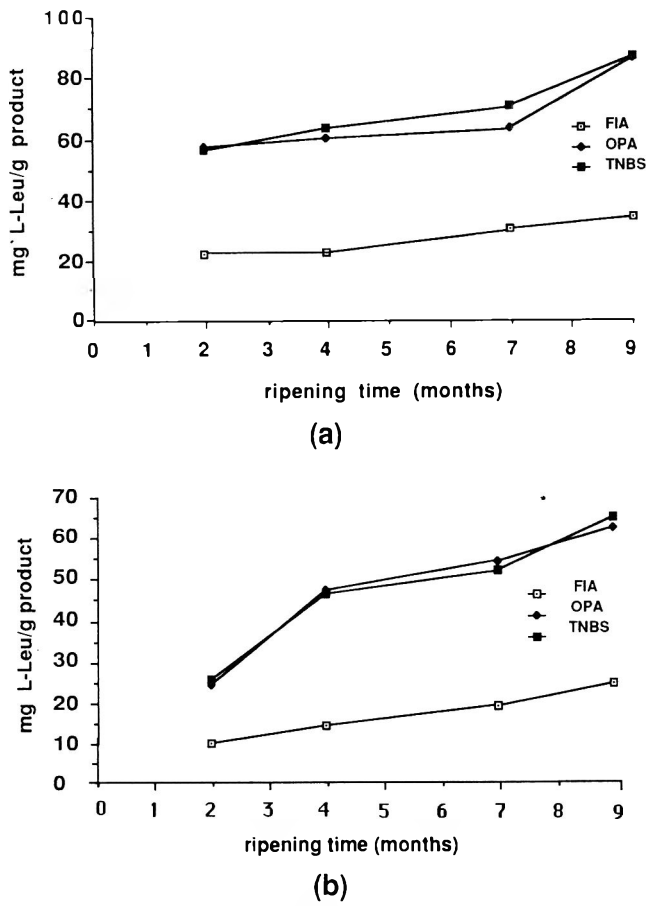

Fig. 3—Free amino acid content in cheese at different ripening times determined by TNBS, OPA and FIA methods. (a) Cheese with added Lactobacillus casei-casei *L2A; (b) control.*

The amino acid content determined by TNBS, OPA and FIA methods in Cheddar cheese with added *Lactobacillus casei-casei* L2A and control are shown in Fig. 3a and 3b. The concentration of free amino acids increased generally with ripening time; values obtained by TNBS and OPA were very close and higher than those obtained by FIA. With FIA no other components such as small peptides caused overestimation of the results. Similar profiles were also obtain with the other samples.

In conclusion, compared to the classical OPA and TNBS methods the proposed FIA method was faster and less time consuming. These advantages would be especially important when a greater number of determinations are required. It was also more sensitive and specific, needed simple instrumentation, and required no sample handling. We recommend the FIA method for measurement of amino acid content as a more specific indicator of the degree of cheese maturity during ripening.

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Determination of Free Amino Acids in Cheese by Flow Injection Analysis with an Enzymic Reactor and Chemiluminescence Detector

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ABSTRACT

The substrate is degraded enzymatically by L-amino acid oxidase immobilized on controlled porosity glass. The hydrogen peroxide generated by the reaction is determined by chemiluminescence with an alkaline reagent containing luminol and hexacyanoferrate (III). The log-log calibration graphs for L-leucine were rectilinear from 0.025 mM to 1.0 mM (R = 0.9998). The coefficients of variation (rds) for n = 10 were 1.4% and 0.3% for 0.1 mM and 0.8 mM of L-leucine, respectively. The sample throughout was 40 h^{-1}. The concentration of free amino acids in cheese samples was 14 to 50 mg L-leu/g product. The enzyme reactor showed good stability over 4-months.

INTRODUCTION

THE MEASUREMENT of amino acids is important in biochemical and industrial analysis (Nanjo and Guilbault, 1974). The concentration of free amino acids in cheese correlates significantly with flavor development and is considered a reliable indicator of ripening (Amantea et al., 1986). Certain specific amino acids are good indicators of proteolytic activity in cheese ripening; methionine, leucine and glutamic acid being most frequently mentioned in the literature (Nasr and Younis, 1986; Marsili, 1985). Many amino acid assay methods have been developed (Church et al., 1983), but most lack sensitivity, are uneconomical and/or time-consuming, e.g. ninhydrin (Reimerdes and Klostermeyer, 1976), *o*-phthaldialdehyde (Church et al., 1983), and enzymatic methods (Lowery and Carr, 1977; Johansson et al., 1976; Guilbault and Lubrano, 1974).

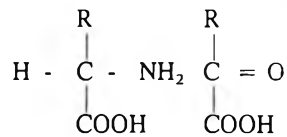
Chemiluminescence (CL) has been widely used analytically because of its high sensitivity, wide dynamic range and simple instrumentation (Burguera and Burguera, 1983). However, because of the intrinsic lack of selectivity of many CL reactions, successful determinations involve coupling the sensitivity of the CL measurement with a step that provides a high degree of selectivity, such as an enzyme reaction (Hansen, 1989). CL determinations of enzymatically generated hydrogen peroxide (H_2O_2) have been reported for many substrates (Murachi and Tabata, 1987; Petersson et al., 1986; Koerner and Nieman, 1986; van Zoonen et al., 1986).

Based on injection of the sample into a nonsegmented carrier stream of a reagent which moves in laminar flow toward and through the detector, the flow injection analysis (FIA) technique does not require complete mixing of sample and reagents or reaching chemical equilibrium. Its principle has been successfully applied to many analytical measurements (spectrophotometric, fluorimetric, chemiluminescence signals, ...) (Burguera and Burguera, 1983). The difficulty of reproducing the physical and chemical conditions in CL can be eliminated by using an FIA system (Petersson et al., 1986; Worsfold et al., 1984; Seitz, 1978), since it provides rapid and reproducible sample injection and mixing with the reagent. Indeed, FIA procedures based on chemiluminescence should prove especially attractive because they have a low detection limit, higher precision and faster sample throughput compared to manual methods of sample processing (Sakamoto-Arnold and Johnson, 1987; Burguera and Burguera, 1983). Several such applications have been described (Kraus and Crouch, 1987; Al-Tamrah et al., 1987; Nussbaum et al., 1987; Ridder et al., 1982).

Several transition metal ions catalyze luminol CL (Kricka and Thorpe, 1983). Cobalt(II) and copper(II) are the most efficient, but ferricyanide was shown to be the cooxidant/catalyst of choice for peroxide analysis coupled to enzymatic assays (Seitz, 1978). Although sensitivity is very high, the background luminescence is unfortunately large. This background level must be carefully measured and kept constant in order to reach a low detection limit. This is readily achieved in an FIA system where the flow is continuously monitored (Rule and Seitz, 1979).

L-amino acid oxidase has been used earlier in enzyme electrodes as it catalyzes oxidative deamination of several L-amino acids according to the following reaction patter:

$$\text{L-amino acid} + H_2O + O_2 \xrightarrow{\text{L-AAO}} \text{2-oxo acid} + NH_3 + H_2O_2$$



The resulting peroxide is then reacted with excess luminol and ferricyanide yielding chemiluminescence

$$H_2O_2 + \text{luminol} \xrightarrow[\text{pH 10.5}]{\text{catalyst}} \text{3-aminophthalic acid} + h\nu$$

The purpose of our study was to develop a sensitive and automated method for rapid determination of free L-amino acids in Cheddar cheese using on-line enzymatic conversion to H_2O_2 and chemiluminescent detection of the H_2O_2 formed.

MATERIALS & METHODS

Materials

L-amino acid oxidase (L-AAO, EC 1.4.3.2, *Crotalus Adamanteus* venom, type V, 1.6 units/mg protein), 3-aminopropyltriethoxysilane (99%), luminol (crystalline, 95-97%), potassium ferricyanide (99.8%), L-amino acids (L-AA21) and controlled pore glass (CPG 240-200) were obtained from Sigma Chemical Company (St-Louis, USA). For the glass, pore size was 242 Å and particle size was 100–200 Mesh. Glutaraldehyde (25%) was obtained from BDH Chemicals Ltd. (Montreal, Canada). All other reagents were commercially obtained and of analytical grade.

The 1.5 mM alkaline luminol working solution was prepared by dissolving luminol in 0.1 M sodium carbonate buffer and adjusting pH to 10.5. The 8.0 mM $K_3Fe(CN)_6$ catalytic working solution was prepared by dissolving the salt in water.

A set of 21 L-amino acids was used as the source of amino acids

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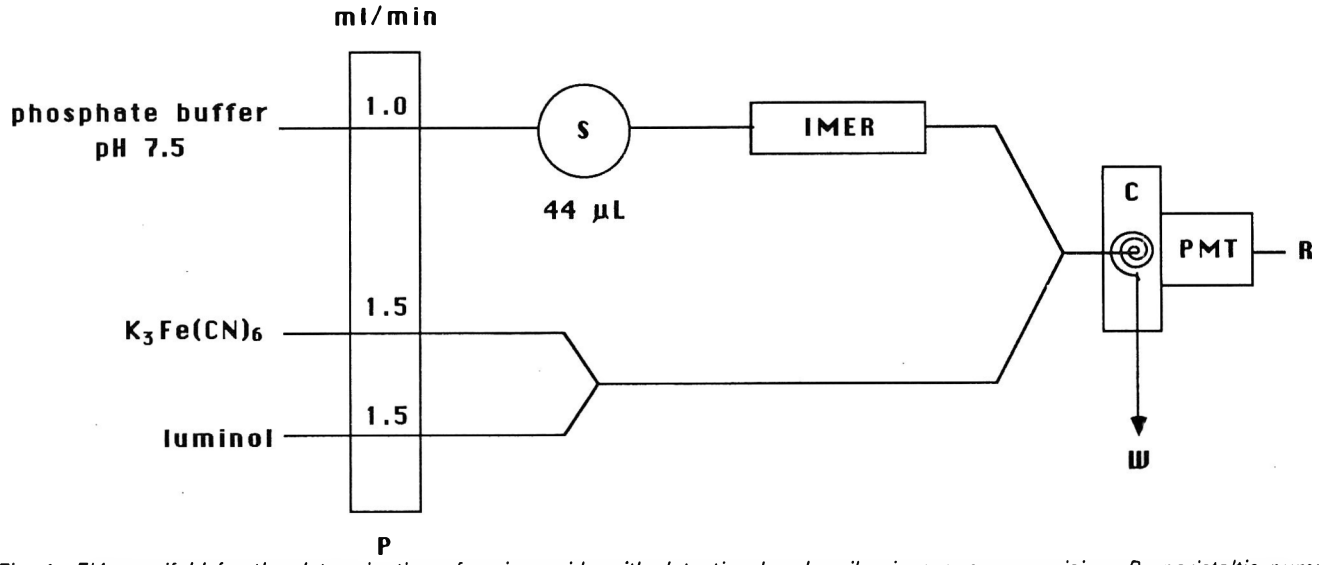


Fig. 1—FIA manifold for the determination of amino acids with detection by chemiluminescence comprising: P—peristaltic pump, S—sample injection, IMER—immobilized enzyme reactor, C—flow cell, PMT—photomultiplier tube, R—recorder, W—waste.

and stored at 0–5°C. Stock solutions of 10 mM of each amino acid were prepared just before use by dilution of the appropriate weighed quantity in 0.1M phosphate buffer pH 7.5, 1.0 mM EDTA. Subsequent dilutions were made with the same buffer to obtain the desired concentrations.

All solutions were prepared with water purified by a Milli Q-system, (Millipore, Bedford, MA, USA).

Procedure

Enzyme immobilization. L-AAO was immobilized onto alkylamine glass beads with glutaraldehyde through Schiff base formation, according to the method of Masoom and Townshend (1984). To 3 mL of the solution of L-AAO was added 0.3g glutaraldehyde-treated glass beads. The solution was kept at 4°C for 2.5 hr, and washed with cold distilled water and cold phosphate buffer to ensure removal of unlinked enzyme.

Protein concentration of the enzyme solution was determined by a colorimetric method (Bradford protein assay, Bio-Rad Labs, Richmond, CA) before and after the immobilization reaction. The coupling yield was then calculated as percent reduction in the amount of protein initially added to the reaction mixture. Immobilization resulted in attachment of 95.9% of the initial protein. The activity of the immobilized enzyme was determined by a batch method (Puchades et al., 1989), using L-leucine as substrate. The specific activity per mg immobilized enzyme was 15% that of the soluble enzyme.

Flow system. The analysis of L-amino acids with chemiluminescence was carried out in the flow system shown in Fig. 1. A four-channel peristaltic pump (Gilson Co., Model Minipuls 3) was used to pump the carrier at 1 mL min^{-1} (0.1M phosphate buffer pH 7.5, 1.0 mM EDTA) and reagents (luminol and hexacyanoferrate(III)) at 1.5 mL min^{-1}. A Rheodyne model 5041 sample injection valve was used to inject a 44-µL sample aliquot into the buffer stream. The sample was carried through the immobilized enzyme reactor (IMER) packed with a wet slurry of the enzyme immobilized on CPG. The IMER consisted of a 7.5-cm long glass column (i.d. 1.7-mm), capped with Teflon end fittings. The catalyst and luminol streams converged, and were mixed with the generated peroxide stream just before the cell. To provide highly efficient collection of chemiluminescence, a special homemade coiled flow cell was employed (Townshend, 1985). This flow-through cell was mounted directly in front of the photomultiplier tube from a Gilson Spectra/glo filter fluorometer, and coiled with reflective aluminum foil to optimize light detection. The signal was recorded on a Perkin Elmer (HP 3390A) integrator and the chemiluminescence peak height measured. All manifold conduits were made from 0.5-mm i.d. Teflon tubing.

Sample preparation. Cheddar cheese was made on a commercial scale by the procedure used by Agropur in 21000L. vats with pasteurized milk (72°C, holding time of 16 sec). Blocks of cheeses were kept at 6°C and 15°C and were sampled at 2, 4, 7, and 9 months maturation. Hydrosoluble fraction was isolated according to the procedure described by Harwalkar and Elliot: (1971), and freeze-dried. Approximately 30 mg of freeze-dried extracts were diluted in 5 mL 0.1M phosphate buffer pH 7.5, sonicated and filtered through a 0.45 µm (Millipore) membrane and a Sep-Pak C_{18} cartridge (Waters Associates, Part No. 51910) to remove fat from the samples.

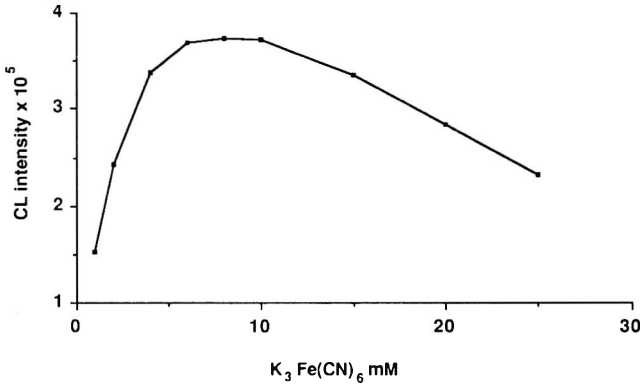


Fig. 2—Chemiluminescence (CL) intensity vs $K_3Fe(CN)_6$ concentration measured in a flow injection system. Conditions: luminol 1.5 mM, pH 10.5 L-leucine 0.5 mM.

Reproducibility of the FIA method. The reproducibility of the FIA method was assessed by analyzing the standard solutions ten times and computing mean values and relative standard deviation (rsd).

RESULTS & DISCUSSION

IN ORDER TO OPTIMIZE the conditions for the reaction, a number of parameters such as reagent composition and concentration, pH, catalyst, and instrumental aspects were investigated using L-leucine as a standard amino acid.

The 0.1M phosphate buffer pH 7.5, 1.0mM EDTA was chosen as carrier according to Barman (1985). When only air-saturated buffer was used, the conversion of L-leucine was found to be incomplete at the tested flow rates. Under such conditions, the oxygen concentration was so low that the enzyme became fully reduced, with a resulting decrease in its turn-over rate. If oxygen was bubbled through the buffer, the efficiency of the reactor increased but depended on the flow rate. In this experiment an oxygen flow rate of 2L min^{-1} was used.

The effect of a catalyst concentration for a 0.5 mM L-leucine solution is shown in Fig. 2. Intensity of the chemiluminescence was very dependent on hexacyanoferrate(III) concentration. At

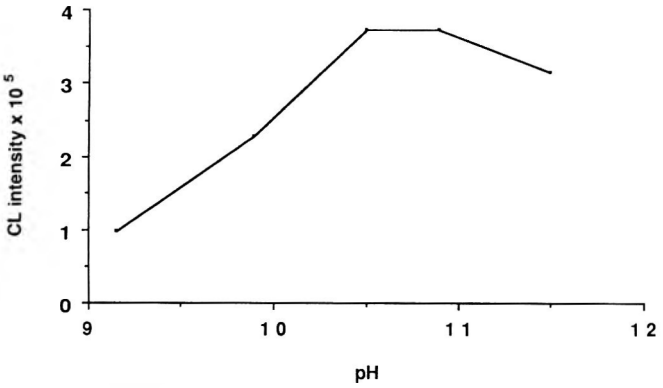


Fig. 3—Chemiluminescence (CL) intensity from 1.5mM luminol solution vs pH using 8 mM ferricyanide as cooxidant catalyst and a 0.5 mM L-leucine standard solution.

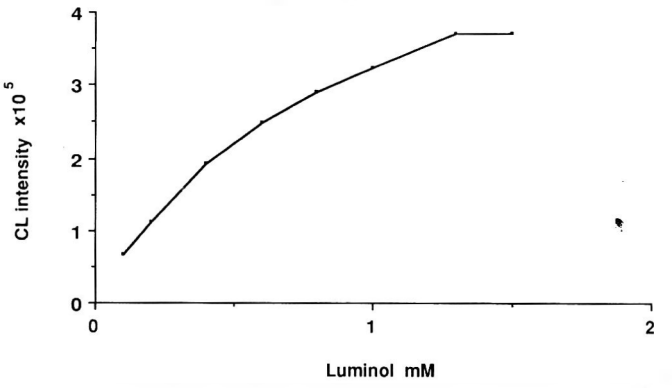


Fig. 4—Chemiluminescence (CL) intensity for an 8mM ferricyanide solution as a function of luminol concentration. Conditions: L-leucine 0.5 mM luminol pH 10.5.

concentrations between 1.0 mM and 8.0 mM, a sharp increase in CL was observed, with the optimum about 7.0 mM followed by a decrease as the level of catalyst was further increased. According to Bostick and Hercules (1975) this diminution could be partly due to the absorption of the emitted light by $K_3Fe(CN)_6$. If copper(II) or cobalt(II) (Kricka and Thorpe, 1983; Klopf and Nieman, 1983) were used, the emitted light was only 11% and 8% that of hexacyanoferrate(III), respectively.

The chemiluminescence of 0.5 mM L-leucine solution was investigated at different luminol pH values. Unbuffered luminol solution (1.5 mM) was used for this experiment. The pH was varied by adjusting the KOH concentration. The concentration of the catalyst was always 8.0 mM. The pH of the luminol was very critical (Fig. 3). At pH less than 9.15, no emission was observed. The greatest CL intensity was found at pH 10.5. The pH range 10.45 to 10.9 should give best precision, since in this range small variations in pH did not significantly affect CL intensity. To ensure the CL intensity was not affected by slight differences in pH, a luminol solution 0.1M with respect to Na_2CO_3 (pH 10.5) was subsequently used.

The intensity of light catalyzed by 8.0 mM hexacyanoferrate (III) as a function of luminol concentration for a 0.5 mM L-leucine solution is shown in Fig. 4. The relative light emission was markedly affected by luminol concentration up to 1.3 mM. At higher concentrations no increase in CL signal was found. A 1.5 mM luminol solution was selected to ensure excess reagent.

The effect of flow-rate was critical. If flow-rates were too low or too high, chemiluminescence was not emitted in the flow cell and hence the signal was not detected. Several different stream flow-rates were examined, and the maximum CL intensities were created at 1.0 mL min^{-1} for carrier and 1.5 mL min^{-1} for luminol and catalyst. These flow-rates were used for all subsequent work. The peak intensities decreased with increased flow-rate for the carrier stream (Fig. 5a). This was a result of reduced residence time in the enzyme column. For the CL reagent streams, the peak intensity increased with flow-rate up to 1.5 mL min^{-1} (Fig. 5b) when a H_2O_2 standard was used.

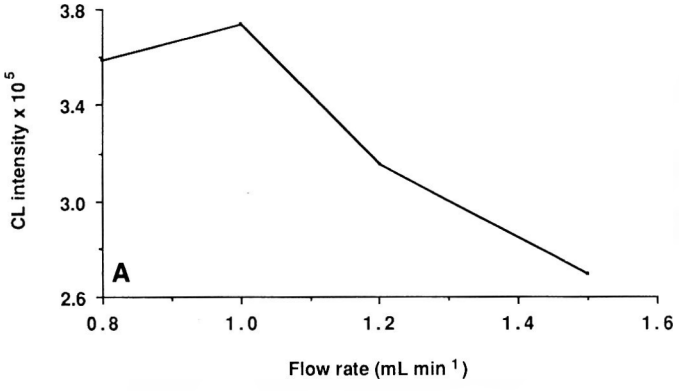
Using the optimized conditions, sample injection volumes from 20-μL to 100-μL were investigated. The CL intensity decreased with smaller sample volumes. From 20-μL to 60-μL the peak height increased markedly with injected volume. Above 60-μL, only a slight increase in peak intensity was observed. Therefore, a 44-μL sample injection volume was used.

System performance

Standard solutions of L-leucine were prepared in 0.1M phosphate buffer pH 7.5, 1.0 mM EDTA, and injected in triplicate. The various parameters optimized as described above were used for FIA calibration. A log-log plot of CL intensity vs L-leucine concentration showed good linearity (correlation coefficient of 0.9998) over a range of 0.025mM to 1.0 mM. The linear part of the resulting calibration graph (Fig. 6) corresponded to the equation

$$\log \text{CL intensity} = 0.96 \log [\text{L-leu}] + 5.82$$

The reproducibility of this procedure was tested by repetitive injections of 10 samples of L-leucine at 0.1 mM and 0.8 mM. The relative standard deviation was 1.4% and 0.3% respectively, adequate for requirements of the method. About 40 samples per hour could be analyzed. When the enzymatic re-



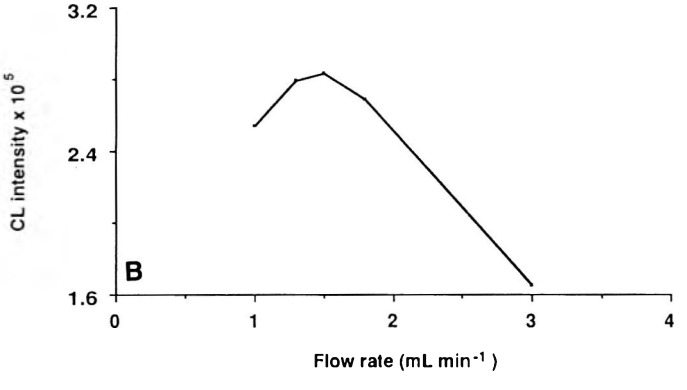


Fig. 5—Chemiluminescence (CL) intensity for 8 mM ferricyanide and 1.5 mM, pH 10.5 solutions as a function of flow rate: (a) carrier; (b) CL reagents.

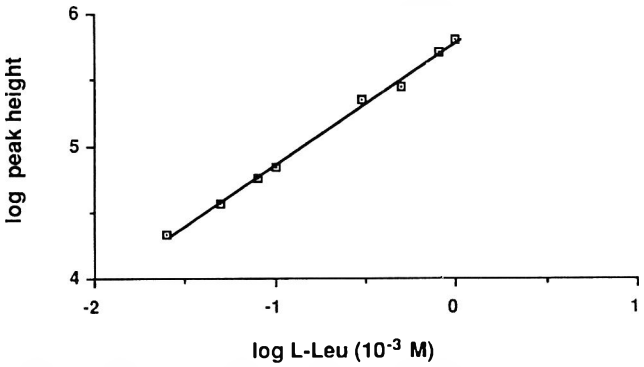


Fig. 6—Log-log calibration curve for L-leucine using a flow injection system.

Table 1—Relative specific activity of L-amino acid oxidase for the L-amino acids

Substrate[a]	Relative activity (%)
L-leucine[b]	100.0
L-phenylalanine	153.2
L-tryptophan	142.1
L-methionine	76.4
L-cystine	48.9
L-tyrosine	42.1
L-cysteine	35.5
L-isoleucine	29.4
L-histidine	14.4
Other L-AA	<6.0

[a] 5.0×10^{-4} M.
[b] L-leucine is used as a reference and is arbitrarily assigned a relative specific activity of 100%.

Table 2—L-Amino acids content of Cheddar cheese samples tested by CL/FIA system

	L-Amino acids (mg L-Leu/g product)[b]					
Sample[a]	T_0	T_1	AT_2	AT_3	$3T_2$	BT_3
1	17.03	26.75	33.64	37.23	42.86	50.52
2	14.07	18.16	22.68	24.70	19.15	27.51

[a] Cheddar cheese
[b] Ripening temperature: A = 6°C, B = 15°C; ripening time months: T_0 = 2, T_1 = 4, T_2 = 7, T_3 = 9.

actor was stored cold (4°C) no loss of activity occurred after a 4-month work period.

Relative activity of the enzyme

The enzyme L-amino acid oxidase is specific for L-amino acids, but is not very selective for the side-chains (Guilbault and Lubrano, 1974). Due to the differences in specific activity of the enzyme for various L-amino acids, the amount of H_2O_2 produced for a given quantity of substrate will depend upon the nature of the amino acid. Therefore the lower limit of detection will vary for each L-amino acid and depend upon the degree of activity exhibited by the enzyme towards that specific amino acid. The activity of the enzyme for each of the 21 natural L-amino acids was measured using the flow system described above, and the relative specific activity for 0.5 mM solution of each amino acid is given in Table 1. L-leucine was used as a reference, and was arbitrarily assigned a relative specific activity of 100%. It has been found that D-amino acids are not acted upon by L-AAO. Other amino acids which showed little or no activity were Gln, His, Arg, Lys, Glu, Val, Ser, Ala and Thr. Substrates with good activity were the L-isomers of Leu, Phe, Trp and Met. These results were in accord with those of Jansen et al., 1988; Lowery et al., 1977; Guilbault and Lubrano, 1974.

Application to cheese samples

In order to apply this method to analysis of L-amino acids in Cheddar cheese, samples subjected to different accelerated ripening treatments and to different storage temperatures were selected. The results are shown in Table 2. It was possible, by this method, to follow the evolution of free amino acids during the Cheddar cheese ripening process.

CONCLUSION

THE CHEMILUMINESCENCE/FIA enzymatic amino acid method described was characterized by short analysis time and good reproducibility. Catalyst and luminol were injected simultaneously into two separate streams and then allowed to merge in a strictly reproducible manner before being mixed with the H_2O_2 produced in the enzymatic reaction. Compared to the classical or *o*-phthaldialdehyde methods, this method was faster, did not require special temperature and had simpler instrumentation. Moreover, it was very specific in contrast to other methods, which are subject to overestimation by measuring other products such as small peptides. Our CL system permits the simple and rapid determination of L-AAs in cheese and other foodstuffs. The adaptation of this system to detection in HPLC seems promising, i.e. for the detection of H_2O_2 produced in post-column enzymatic reactors.

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Recovery of Proteins from Raw Sweet Whey Using a Solid State Sulfitolysis

JUAN M. GONZALEZ and SRINIVASAN DAMODARAN

ABSTRACT

A simple, efficient method to recover functional proteins from cheese whey was developed. It involves partial sulfitolysis of disulfide bonds in whey proteins using sodium sulfite and solid state copper carbonate catalyst. About 25 to 40% of the initial disulfide bonds were modified within 15 to 30 min at pH 7.0. When treated whey was adjusted to acidic pH, about 70 to 80% of the whey proteins precipitated below pH 5.0. The protein precipitate contained some copper as protein-copper complex. However, extraction of the precipitate with EDTA at pH 4.5 completely removed the copper. The uncomplexed protein showed a U-shaped pH-solubility profile with 100% solubility below pH 3.0 and above 6.0. About 90% of the proteins precipitated in the pH range 4.0 to 5.0. The method could be used on an industrial scale to recover highly functional and nutritional whey protein for use in a variety of products.

INTRODUCTION

WHEY is a by-product of the cheese industry. An enormous amount of whey is produced worldwide. The *1988 World Dairy Situation Report* of the International Dairy Federation showed approximately 12.2 million metric tons of cheese was manufactured annually. Projected calculations (8.5 kg whey/kg cheese) indicated nearly 104 million metric tons of raw whey was produced annually. Assuming the protein content of raw sweet whey at about 0.6%, almost 620 thousand metric tons of whey protein could be produced, and, if available, could help alleviate world protein shortages. However, most whey generated around the world is disposed of as waste. In the USA alone 40% of the production is disposed as raw whey (Zall, 1984).

Whey proteins are far superior to other food proteins in terms of nutritional quality, especially lysine and methionine (FAO, 1973). Recovery of whey proteins and their use in human foods might also help reduce cost of cheese production. Although the potential of whey protein use in human foods is very clear, the major obstacles at present seem to be twofold: (1) the economics of its recovery, and (2) the functional properties of isolated whey proteins.

Methods available to prepare whey protein isolate (> 90% protein) are either uneconomical and/or the proteins isolated by existing methods lack critical functional properties such as foaming, emulsification or gelation. The isoelectric precipitation method would be the simplest of all methods for recovering protein from a dilute solution of whey. However, this approach has been ineffective with whey because the proteins are highly soluble even at their isoelectric point. The high solubility of whey proteins is mainly related to their unique molecular properties. Even when electrically neutral, the strength of their interaction with water is stronger than among themselves; this renders them soluble, even at their isoelectric pH. However, this strong affinity for water can be decreased and the affinity between protein molecules can be increased by slightly altering the protein structure.

Subtle alterations in protein conformation can be achieved by cleaving one or a few disulfide bonds. Since the major whey proteins (i.e., serum albumin, β-lactoglobulin and α-lactalbumin) contain several disulfide bonds, cleavage of a few would alter their conformation in solution and affect their pH-solubility profiles. Recently, we developed a novel solid state catalyst method to systematically cleave disulfide bonds in proteins under nondenaturing conditions (Gonzalez and Damodaran, 1990). The method is based on a reduction-oxidation reaction system depicted as follows:

$$RS - SR + SO_3^{=} \rightleftarrows RS - SO_3^{-} + RSH$$

(Oxidizing agent)

where RS-SR represents the disulfide bonds. Addition of sulfite to the protein initially cleaves the disulfide bonds resulting in formation of one S-sulfonate derivative and one free SH group for each disulfide bond. In presence of an oxidizing agent, such as copper, the free SH groups can be oxidized back to disulfide bonds, which are again cleaved by the sulfite present in the system. The reaction cycle repeats until all disulfide bonds in the protein are converted to the $S\text{–}SO_3^{-}$ derivative.

The objective of our study was to use a solid state copper catalyst column reactor to partially cleave disulfide bonds in whey proteins, which could then be recovered by precipitation at pH 4–5.

MATERIALS & METHODS

Materials

Raw sweet whey was obtained from Babcock Hall Dairy Plant (Food Science Department, University of Wisconsin-Madison). Whey was centrifuged at 3,000x*g* to separate remaining casein particulates. Sodium azide (0.02%) was added to the centrifuged whey to prevent microbial spoilage. The whey was then stored at 4°C for no longer than 36 hr. Copper carbonate attached to silica beads (4–10 mesh, 6% Cu) was obtained from Alfa Products, Thiokol/Ventron Div. (Denvers, MA). Bovine serum albumin (BSA) and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co. (St. Louis, MO). Whey protein isolate (WPI) was prepared by dialysing a 5% solution of commercial whey protein concentrate (Daritek 35 from Foremost Whey Products, Baraboo, WI) vs water for 72 hr to remove lactose and minerals. The dialysed solution was lyophilized. Soy protein isolate was prepared from defatted soy flour (Central Soya, Chicago) according to the method of Thant and Shibasaki (1976). All other chemicals were reagent grade.

Sulfitolysis of sweet whey

The packed column reactor system for sulfitolysis of sweet whey is shown in Fig. 1. About 1.5g of pre-washed copper carbonate-silica bead (containing about 6% copper) was packed in a glass column (15 cm length and 0.5 cm internal diameter). The bottom end of the tube was covered with a nylon cloth and connected to an Amicon LP-1 peristaltic pump using Tygon® tubing. The bottom end of the column was placed into a water-jacketed beaker. A Tygon® tube was connected to the inlet end of the peristaltic pump and the other end of the tube was immersed in the water-jacketed beaker. Twenty-five mL of raw sweet whey (RSW) were placed in the temperature-controlled container. Sodium sulfite was added to 0.1M and the pH of the system adjusted to 7.0. The reaction was initiated by circulation of whey through the column. The flow rate was maintained at 0.67 mL/sec. Aliquots of whey (0.35 mL) were withdrawn at various intervals and

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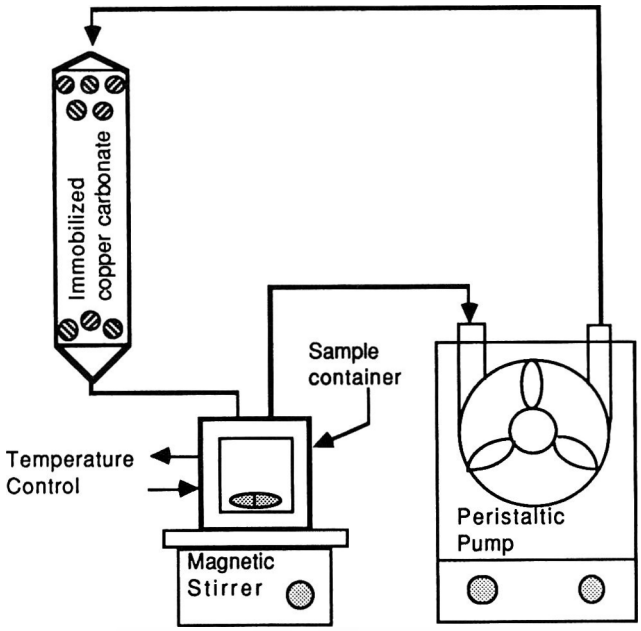

Fig. 1—Schematic of reaction system for oxidative sulfitolysis of proteins in raw sweet whey using $CuCO_3$-silica gel bead catalyst.

immediately centrifuged to separate loose copper carbonate particles in solution. These aliquots were analyzed for disulfide and sulfhydryl groups remaining as a function of time. At least triplicate measurements were made. The error within triplicates was negligible.

Protein determination

Preliminary investigations on protein estimation using the Lowry and biuret methods gave unreliable results, probably due to interference from nonproteins and minerals. To overcome these, the protein estimation was performed as follows: First, a standard curve of concentration versus absorbance at 280 nm for BSA was prepared. The absorbance of whey at 280 nm was measured and the protein concentration was calculated from the BSA standard curve. The protein content of sweet whey estimated by this method ranged form 0.65–0.85%, which is close to reported values (Zall, 1984).

Determination of S-S and SH

Disulfide and sulfhydryl of whey were determined by the 2-nitro-5-thiosulfobenzoate (NTSB) method (Thannhauser *et al.*, 1984; Damodaran, 1985). Since several proteins with different S-S and SH were present in whey, the S-S and SH were expressed as number of moles per 43,000 gmole of protein. Based on this RSW contained about 6 moles S-S and SH per 43000 gmole of protein. The extent of sulfonation of S-S and SH during the course of the sulfitolysis reaction was calculated by subtracting the remaining S-S + SH (as a function of reaction time) from the total S-S + SH of RSW at zero time.

Solubility

The pH-solubility profile of sulfonated proteins was determined as follows: The sulfonated whey was adjusted to various pH values using either 4N HCl or NaOH. The samples were centrifuged in an Eppendorf microcentrifuge 1 min. Protein concentration in the supernatant was determined by measuring absorbance of the supernatant at 280 nm. Solubility at each pH was calculated as the ratio of absorbance at 280 nm of the treated sample to that of the RSW.

Copper estimation

Copper in the sulfonated whey protein was determined using an atomic absorption spectrophotometer (Perkin Elmer, Model 2380). The total copper of sulfonated RSW was obtained by directly analyzing the centrifuged sample withdrawn from the reaction vessel.

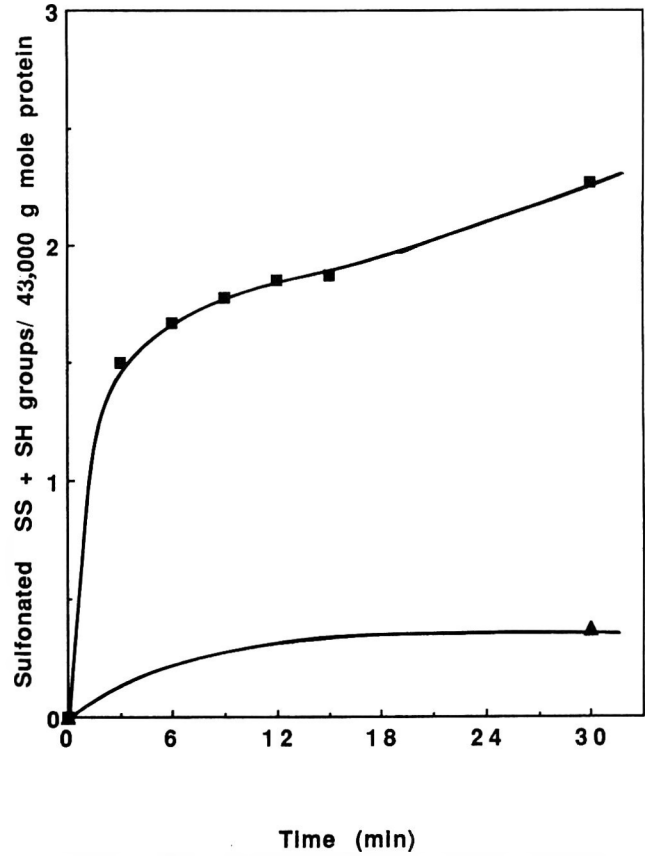

Fig. 2—Sulfonation of S–S and SH groups with time during sulfitolysis of raw sweet whey using $CuCO_3$-silica gel bead catalyst. Conditions: pH 7.0, 0.1M Na_2SO_3, 25°C, ▲-▲, control, no copper catalyst; ■-■, with copper.

RESULTS

THE PROTEIN of the whey varied from 0.65% to 0.85%, and initial pH of RSW was about 5.2. The molecular weight of 43,000 for whey protein we used is based on the average molecular weight of the proteins in whey, calculated from the % distribution of various whey proteins (Marshall, 1986).

The time course of oxidative sulfitolysis of disulfide bonds of proteins in RSW is shown in Fig. 2. When whey containing 0.1M Na_2SO_3 without copper catalyst was incubated at 25°C, about 0.4 moles of S–S + SH per 43000 gmole of protein was sulfonated after 30 min. This slight amount in absence of a catalyst may be attributed to the native redox potential of RSW. When the whey containing 0.1M Na_2SO_3 was circulated through the copper catalyst column, about 1.5 moles of S–S + SH were sulfonated within 3 min; after the initial 3–6 min the extent of sulfonation did not further increase significantly with time (Fig. 2).

Previous studies on sulfonation of proteins using solid copper carbonate catalyst indicated the sulfonated proteins could chelate copper (Gonzalez and Damodaran, 1990). To know the amount of copper chelated by whey components, the copper content of the sulfonated whey was determined. As shown in Fig. 3, the copper of sulfonated whey increased with reaction time, indicating copper from the copper-carbonate-silica bead was solubilized as whey proteins were sulfonated. To determine whether solubilized copper was present as free, copper or as a protein-copper complex, proteins from the whey samples were precipitated by adjusting the pH to 4.5, and then separated by centrifugation. The pellet was dissolved in dilute NaOH and the volume made up to the original volume. The copper content of this protein fraction is shown in Fig. 3. We noted while the total copper content of whey increased with reaction time, the copper associated with the precipitable pro-

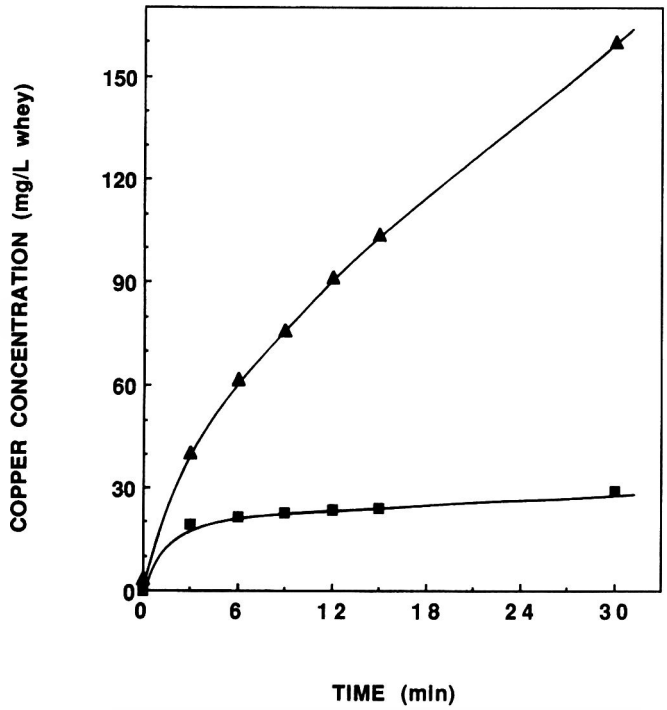

Fig. 3—Total copper of whey (▲-▲) and amount of copper associated with the protein fraction (■-■) as a function of sulfitolysis time. Conditions same as Fig. 1.

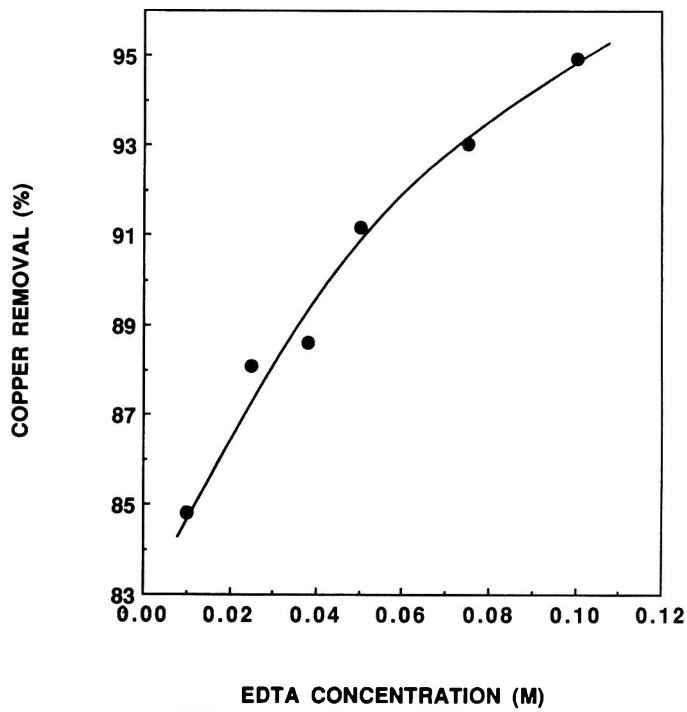

Fig. 5—Effect of EDTA on removal of copper from sulfonated whey protein precipitates at pH 4.5. Initial copper was 0.7–1.0 mg/g protein.

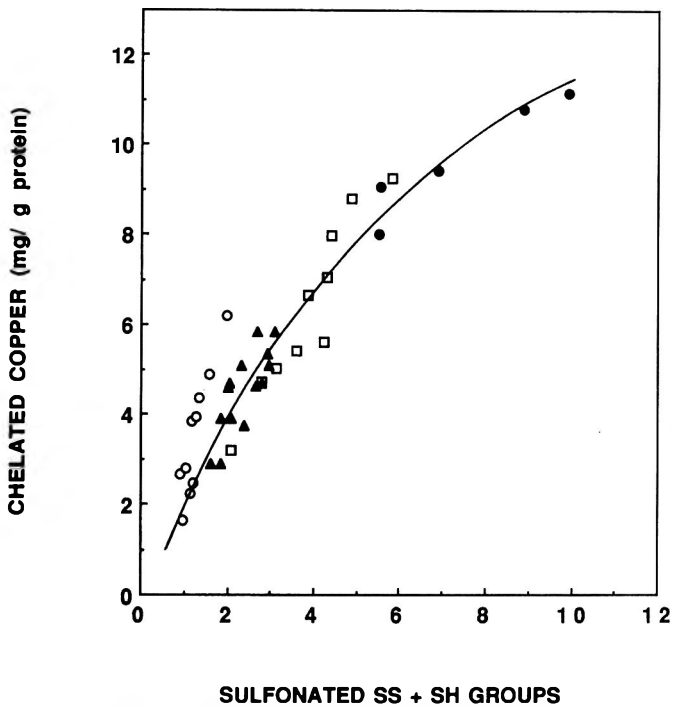

Fig. 4—Relationship between copper chelated by proteins and number of S–S + SH groups sulfonated during sulfitolysis of various proteins. Conditions: 1% protein solutions, pH 7.0, 0.1M Na_2SO_3, 25°C. Key: □, whey protein isolate; ●, bovine serum albumin, ▲, soy protein isolate; 0, RSW.

tein reached a maximum value at about 3–6 min and remained constant. We also noted that the number of S–S and –SH groups sulfonated reached a maximum within 3–6 min (Fig. 2), indicating the copper complexation by protein was related to sulfonation. The data in Fig. 3 indicated at 6 min, when sulfonation was nearly maximum, about 35% of the total solubilized copper was in the precipitable protein fraction. The remaining copper may have been either in free form or complexed with other whey components such as low-molecular-weight peptides and acids such as lactic, citric and acetic generated by starter bacteria from cheese production.

To determine whether copper chelation by whey proteins was due to nonspecific binding to the protein or binding to sulfonated groups various food proteins were sulfonated and the relationship between the number of S–S + SH sulfonated and the copper content was examined. The copper chelated by various proteins increased with the number of S–S + SH sulfonated (Fig. 4). The fact that data obtained with three different protein sources fell in a single curve strongly suggests copper chelation was not dependent on specific properties of proteins, but solely related to sulfonated groups introduced during the sulfonation reaction.

To determine whether the copper from sulfonated proteins could be efficiently removed by treatment with EDTA, the effect of EDTA concentration was studied. The sulfonated RSW was adjusted to pH 4.5 to precipitate proteins, and centrifuged. The pellet was resuspended in EDTA at pH 4.5, the suspension stirred 3 min and centrifuged. The percent removal of copper by EDTA was calculated from the ratio of copper in the protein after and before EDTA treatment. The percentage of copper removal from the WPI-copper complex as a function of EDTA concentration is shown in Fig. 5. A single extraction with 0.01 M EDTA removed about 85% of copper initially present. About 95% of the copper was removed when treated with 0.1M EDTA. These results indicated copper chelated to sulfonated proteins could be removed effectively.

The pH vs protein solubility profiles of RSW and sulfonated sweet whey are shown in Fig. 6. With RSW the solubility of proteins remained above 90% in the pH range 2.0–8.0. However, when about 2 moles of S–S + SH were sulfonated, the resultant sulfonated protein-copper complex exhibited an *S*-shaped pH-solubility profile. About 70% of the whey proteins precipitated from the sulfonated whey at pH 4.0; however, the precipitated protein-SO_3-Cu complex remained insoluble when the pH was further decreased to 2.0. When copper was removed from the sulfonated whey proteins by treatment with 0.1M EDTA, the resultant copper-free protein exhibited a U-

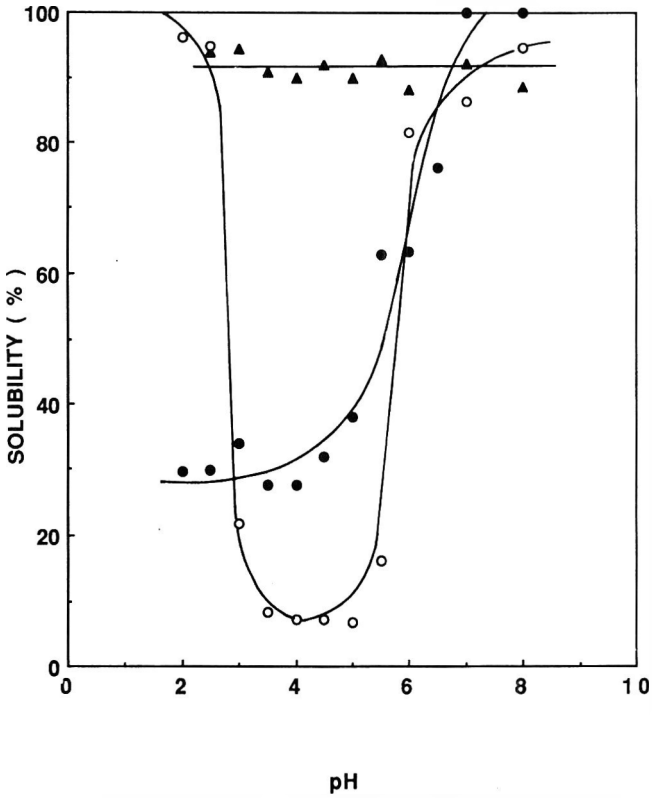

Fig. 6—The pH vs protein solubility profile of (▲) raw sweet whey; (●) sulfonated sweet whey (6 min); and (0) sulfonated sweet whey protein after precipitation at pH 4.5 and extraction with 0.1M EDTA at pH 4.5.

shaped pH-solubility profile. While the solubility was more than 90% above pH 6.0 and below pH 3.0, about 90% of the whey proteins precipitated between pH 4–5 (Fig. 6). The data indicate whey proteins can be induced to precipitate at pH 4.5 by causing limited cleavage of disulfide bonds (i.e., about one disulfide bond per 43,000 gmole of protein) using sulfonation. This approach could be used to recover functional proteins from sweet whey.

DISCUSSION

THE SIMPLEST WAY of recovering protein from whey is isoelectric precipitation and several other chemical precipitation methods have been developed in the past. These included precipitation with ferric salts (Imado et al., 1962; Amantea et al., 1974), ferripolyphosphates (Jones et al., 1972), tannic acid (Murray, 1968) and polyacrylic acid (Sternberg et al., 1976). Although these methods were effective in precipitating whey protein at pH 3–4, the resultant isolate usually contained high levels of the added salts. For example, the composition of whey protein precipitated with ferripolyphosphate was 22% protein, 12% iron and 39% phosphate (Jones et al., 1972). The high levels of phosphate in these preparations could pose nutritional safety problems, for instance, kidney dysfunction infants (Amantea et al., 1974). Furthermore, the ferric-protein obtained from the ferric chloride precipitation method (Jones et al., 1972) usually causes brown discoloration from the bound ferric ion.

Because of the sensory and possibly certain nutritional safety problems associated with the salt-precipitated whey proteins, attempts were made to precipitate whey proteins with carboxymethylecllulose and other anionic polysaccharides (Hidalgo and Hansen, 1969, 1971; Hansen et al., 1971). These studies showed the whey proteins could be precipitated by carboxymethylcellulose (CMC) at pH 3.2. The protein from the insoluble protein-CMC complex could then be separated by adding calcium which precipitated CMC as insoluble calcium salt (Hill and Zadow, 1978). To overcome the operational difficulties involved in the CMC precipitation method, and the loss of significant, costly CMC during the process, in recent years research efforts have been directed toward ion exchange adsorption to recover proteins (Mirabel, 1978; Nichols and Morr, 1985; Barker and Morr, 1986). The major steps are: (1) initial adsorption of proteins to the ion exchange resin by passing whey through the column; (2) elution of the adsorbed proteins by eluting with NaCl or dilute alkali, and (3) concentration and drying the final protein. Although the ion exchange adsorption process provided good-quality whey protein, the method had high capital cost and is not very practical at the industrial scale (Barker and Morr, 1986).

Heat-precipitation of whey protein at low pH, i.e., at pH 4.5, has been successful (Hill et al., 1982). However, heat-precipitation caused extensive denaturation, decreased solubility and impaired various functional properties. these deleterious effects on protein functionality effectively preclude usage of that protein in many formulated foods. In fact, whey protein obtained by heat-precipitation has been useful only in ricotta cheese making (Hill et al., 1982).

At present the method of choice for recovering proteins from whey is ultrafiltration/diafiltration (McDonough et al., 1974; Breslau et al., 1975). However, the whey protein concentrate (WPC) obtained by ultrafiltration contained about 35–70% protein. The solubility of commercial WPC is only about 70%, owing to partial denaturation of the proteins. The high lactose content and poor functional properties may be major factors limiting extensive use of WPC in food products. Hence, efforts to develop alternate recovery processes that can be carried out at small or medium-size cheese manufacturing centers are desirable.

Our results indicate the pH-solubility profile of whey proteins can be altered by opening one or two disulfide bonds, per 43,000 gmole protein using sulfonation. The method was simple, novel and could be carried out with lower capital cost in small cheese manufacturing centers. The only additive used was sodium sulfite, which is FDA-approved. Moreover, when the sulfonated protein was removed by precipitation at pH 4.0–4.5, virtually all sodium sulfite remained in the supernatant. Although 0.1M Na_2SO_3 was used in our study, the process could be carried out with Na_2SO_3 concentration as low as 0.02M (Gonzalez and Damodaran, 1990); however, at that low concentration, the time to cleave disulfide bonds may be longer than 6 min. Although raw sweet whey was used in our study, whey preconcentrated (about 50 to 70% water removal) by ultrafiltration could be used as feed for this process. This approach would drastically decrease the amount of Na_2SO_3 required and also increase the life of the copper carbonate-silica bead column reactor.

Even though copper carbonate is insoluble in water (*Handbook of Chemistry and Physics*, 1985), a significant amount of copper was chelated by the protein upon sulfonation. However, treatment of the protein-Cu complex with low concentrations of EDTA effectively removed it (Fig. 5). Multiple extraction or countercurrent extraction of the protein precipitate with EDTA solution may require much lower EDTA and enable preparation of copper-free protein isolate.

The sulfonated whey protein should not pose a nutritional safety problem, because only one or two moles of sulfonate ($-SO_3^-$) group would be added per 43000 gmole of whey protein. Furthermore, the $S-SO_3^-$ bond was acid-labile. Under the acidic condition of the stomach, the sulfonated protein would be desulfonated and the liberated cysteine residues should be biologically available. Since copper-catalyzed sulfonation is very specific to cysteine and cystine residues, the bioavailability of other essential amino acids, such as lysine and methionine, would not be impaired. Other chemical modification methods such as acylation with acid anhydrides predominantly

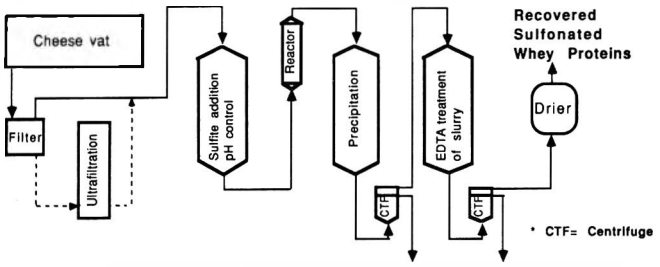

Fig. 7—Flow chart for isolation of proteins from raw sweet whey by sulfitolysis.

involve irreversible modification of lysyl residues, and to a lesser extent histidine and cysteine residues, which decreases their bioavailability.

Based on our results presented, a simple process for recovering proteins from raw sweet whey is schematically shown in Figure 7.

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Use of Bicarbonates for Microbial Control and Improved Water-Binding Capacity in Cod Fillets

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ABSTRACT

Dipping cod fillets in ammonium or sodium bicarbonate solutions markedly reduced microbial growth compared to untreated samples after 8 days at 4°C. Total plate counts, proteolytic bacteria and H_2S-producing bacteria were inhibited. Mixing ammonium- and sodium bicarbonates at selected ratios maintained the preservative effect while reducing the ammonia odor. The treated fish had improved texture and moisture retention by both objective and subjective testing but had significantly lower aroma and overall acceptability scores from sensory evaluation.

INTRODUCTION

MOST SPOILAGE of fish is bacteriological; the changes that take place are mainly due to bacterial enzymes. The complex changes associated with rigor mortis are due to fish muscle enzymes as is the progressive breakdown of nucleotides and nucleosides (Kassensarm et al., 1963). In the absence of bacterial activity, these do not lead to spoilage. Sterile fish flesh can be held under refrigerated conditions up to 6 wk without becoming spoiled (Shewan, 1977). Fish held without such precautions allow growth of psychrotrophic bacteria which secrete enzymes into the tissues, causing quality loss.

Chemical preservation of fish has been extensively studied. Potassium sorbate dips were of minimal value in controlling microbial flora of red hake and salmon (Fey and Regenstein, 1982). In English sole, treatments with 0.1 and 1.0% potassium sorbate extended the microbial lag period to 1 and 6 days, respectively (Chung and Lee, 1981). Combining chemical preservation with modified atmosphere packaging has met with some success. Dipping the Australian fish morwong in a combined solution of 1.2% potassium sorbate and 10% polyphosphate, followed by packaging in 100% CO_2 extended shelf life (Statham et al., 1985). Miller and Brown (1984) reported dipping rockfish fillets in 1% potassium sorbate plus 5 ppm chlortetracycline, followed by vacuum packaging and storage at 2°C, retained freshness after 14 days storage. Woyewoda et al. (1984) used both modified and controlled atmospheres of 60% CO_2/40% air and storage at 1°C, but found no difference between treated cod and controls. Fillets packaged in modified atmosphere, 50% Co_2/50% O_2 and stored at 2°C had acceptable shelf life of 14 days (Stenstrom, 1985).

Since sodium bicarbonate (SB) and ammonium bicarbonate (AB) impart antimicrobial activity in some systems, their use to extend shelf life of cod fillets was evaluated. SB inhibited growth of food-related bacteria and yeast in model systems (Corral et al., 1988). AB and SB also inhibited spoilage of apple juice by *H. wingei* and *S. cerevisiae*, but caused off-flavor and darkening of the juice at effective concentrations (Curran and Montville, 1989). A potential problem associated with bicarbonate usage is that an increase in pH might affect the overall microbial ecology, enabling spoilage by other organisms. Because of this problem in high acid foods, the inhibitory effect of bicarbonates in a more alkaline food with better buffering capacity was evaluated. Cod fish was chosen for our study because its postmortem pH is generally neutral and because of its economic importance. Our objective was to determine whether SB and AB could be used to control microbial growth on cod, and to improve its texture. We also determined overall acceptability scores to see if they were significantly lower than untreated fish due to residual ammonia flavor and odor.

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MATERIALS & METHODS

Treatment of fish

The effect of bicarbonate treatments on bacterial growth in cod fillets was examined by dipping cod fillets (obtained locally at the retail level) in saturated SB (8%), saturated AB (12%), a distilled water control, or distilled water adjusted with 6N NaOH to the pH of saturated SB (pH 7.9) for 30 min. The cod was placed in loosely sealed stomacher bags, stored at 4°C and sampled on days 0, 1, 2, 3, 4, 6, and 8.

Microbial analysis

Sampling consisted of stomaching 50g fish with 450 mL cold 0.1% peptone water for 2 min. Total bacterial growth was determined by standard plate counts in duplicate using Plate Count Agar (PCA) (Difco Laboratories, Detroit, MI). Plates were incubated 4 days at 20°C. Hydrogen sulfide producers, indicators of odor-producing organisms, were enumerated on H_2S media (Ogden, 1986) after 4 days at 20°C. Proteolytic bacteria, which cause structural deterioration of fish, were counted on media by the zone clearing method of Levin (1968).

Mixtures of AB and SB were examined because, although AB was more effective than SB, it imparted a strong ammonia odor. Solutions of saturated AB and saturated SB were prepared in the following AB:SB ratios: 1:1, 1:3, 1:7, and 1:15. Controls consisted of saturated AB alone and distilled water. The cod were dipped for 30 min in the solutions and stored at 4°C. Total aerobic plate count was determined on days 0, 2, 4, 6, and 8.

Texture measurements

The treated fish had obvious texture changes which were quantified by determining centrifugal drip (CD) and cooked drip (CKD) of triplicate samples. These strongly correlated to texture measurements in fish (Krivchenia and Fennema, 1988). Raw, ground samples were centrifuged for 10 minutes at 200 rpm in a Sorvall RC-5B centrifuge (Dupont, Chadds Ford, PA). CD was the % moisture lost from the original sample. Cooked drip values were measured by placing 50g of cubed raw fish in Ziplock® bags and immersing them in an 80°C water bath for 30 min. The juice lost during cooking was then weighed. Objective texture measurements were made using an Instron texture testing instrument equipped with a Kramer shear-compression cell. Chart and crosshead speed were 250 mm/min. Triplicate samples of cooked fish were drained, diced, placed randomly in the Kramer cell, and sheared once. In all texture measurements, triplicate values were averaged and 95% confidence intervals determined using Student's t test (Freund, 1967).

pH determinations

To determine if differences in pH could account for different texture properties of treated cod, the pH of triplicate fish samples was determined using the pH dilution method of Mizushima et al. (1964).

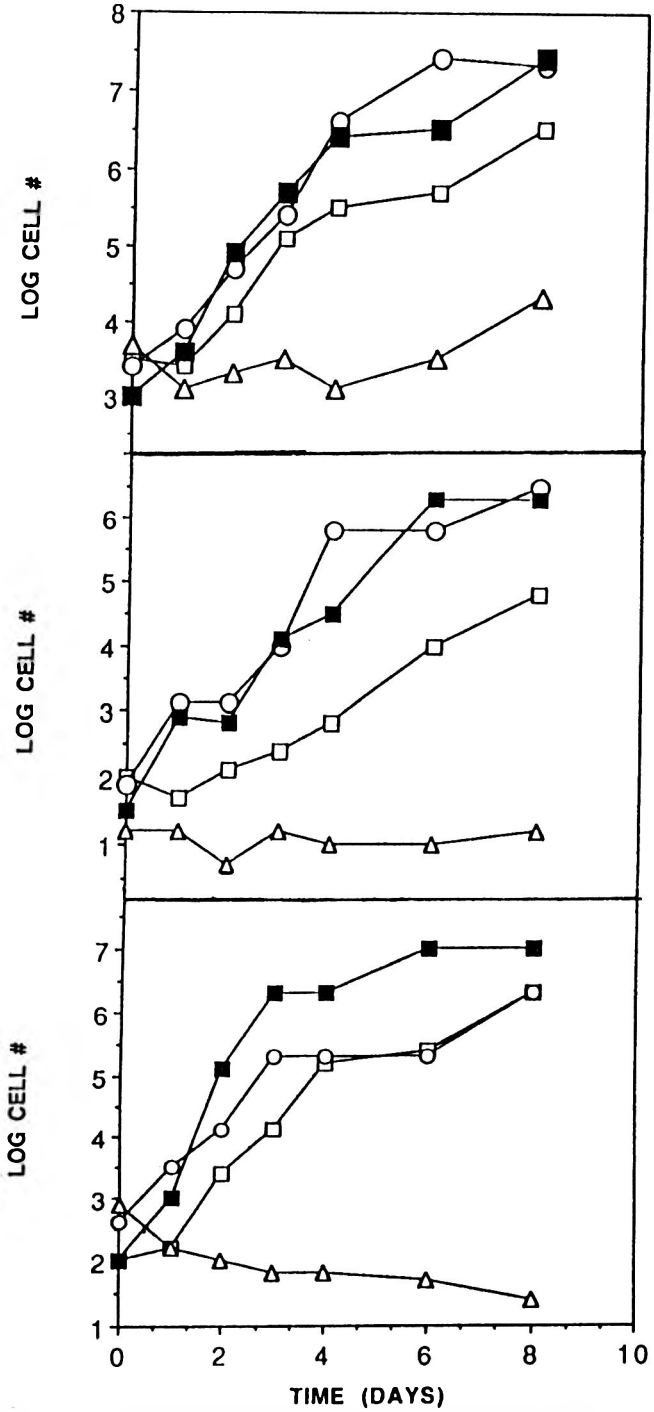

Fig. 1.—Total aerobic growth (top panel), hydrogen sulfide producers (middle), and proteolytic organisms (bottom) on cod fillets treated with saturated sodium bicarbonate (□), saturated ammonium bicarbonate (△) pH-adjusted distilled water (○), and distilled water (■).

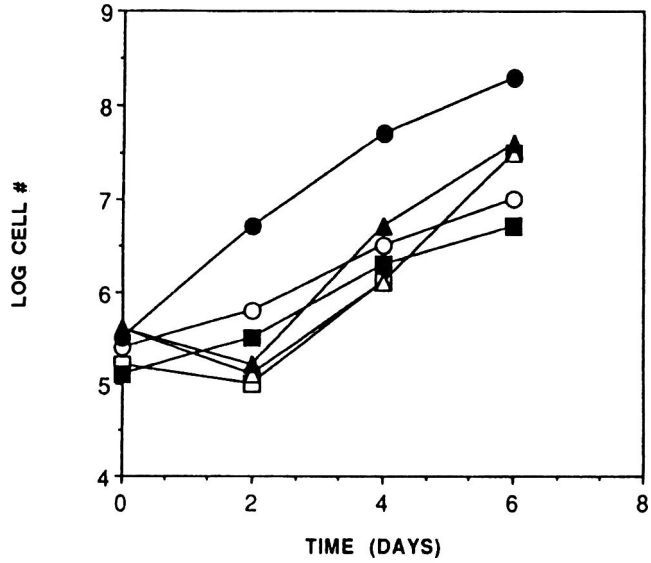

Fig. 2.—Total aerobic growth on cod fillets treated with saturated ammonium bicarbonate (□) and saturated ammonium bicarbonate with saturated sodium bicarbonate prepared in the following ammonium bicarbonate:sodium bicarbonate ratios: 1:1 (△), 1:3 (○), 1:7 (■), 1:15 (▲), and distilled water (●).

Sensory evaluation

Sensory tests were done by 30 subjects recruited from students and staff of the Food Science Department. The subjects had previously participated in taste panels and were familiar with the use of the rating scales. Samples were evaluated for appearance, aroma, moistness, flavor and overall acceptability using a 15 cm. unmarked horizontal line scale anchored at both ends with the phrases "like extremely" and "dislike extremely". Ratings were quantified by measuring in mm the distance of the panelist's mark from the origin of the scale.

Fillets were dipped for 30 min in either a 1:15 mixture of saturated AB to SB (1:15), a solution of saturated sodium chloride (NaCl) or distilled water (DW). Sodium chloride treatment was used as control for the salty taste of the 1:15 treated fish. Samples were stored at 4°C for 0 and 3 days. Fillets were baked on uncovered trays in a conventional oven at 350° F for 20 min. The edges of each fillet were trimmed and discarded and the remaining portion was cut into 1 cm^2 pieces. Twenty-eight gram samples were placed in plastic souffle cups, covered and held in a warming oven prior to the evaluation. Subjects evaluated the samples in a single 20-min session. Data were analyzed by analysis of variance (SAS, Version 5, SAS Institute, Cary, NC) and differences between treatment means were evaluated using the least significant difference test.

RESULTS & DISCUSSION

THE MAJOR SPOILAGE BACTERIA of fish are in the genera *Pseudomonas* and *Achromobacter* (Chai et al., 1968), most species of which produce H_2S (Ogden, 1986). AB greatly inhibited H_2S-producing bacteria, proteolytic organisms, and total bacterial growth on cod (Fig. 1). Total growth on fillets treated with AB had a delayed lag phase, and final numbers were approximately 1000-fold lower than the untreated control. If, as suggested by Shipman and Wyler (1989), 10^6 cells/gram constitutes microbial spoilage, the untreated fish and pH-adjusted controls spoiled after 4 days. Previous work in our laboratory demonstrated the bicarbonate effect was independent of cation and not due to sodium or ammonium (Corral et al., 1988; Montville and Goldstein, 1987). SB-treated fish reached 10^6 cells/gram after 7 days. AB-treated fish remained microbially stable for the entire storage period.

Hydrogen sulfide producers were completely inhibited for the entire storage period by the AB treatment (Fig. 1, middle panel). The controls reached 10^6 cells/gram within 6 days, but the SB- treated fish did not, even after 8 days. Proteolytic organisms were also completely inhibited for the entire study by the AB treatment (Fig. 1, bottom panel). The untreated fish reached 10^6 cells/gram in 3 days, but the SB and pH-adjusted control treatments did not reach this level until after 7 days. In this case, the action of the SB (but not AB) appeared pH-related.

Although AB was more effective than SB in inhibiting microbial growth, the ammonium remaining in the fish caused an ammonia off-odor detectable immediately after dipping. Decreasing concentration of AB did not eliminate the problem, so use of mixtures of AB and SB was examined. While these were effective, the effects of the mixed bicarbonate dips were not as dramatic as the earlier study due to the higher initial

Table 1—Drip and texture measurements of cod fillets dipped in water, 12% ammonium bicarbonate or saturated ammonium bicarbonate:saturated sodium bicarbonate solutions at ratios of 1:1, 1:3, 1:7, and 1:15

Treatment	Centrifugal drip (% expressable moisture)	Cooked drip (% wt loss)	Instron texture measurement (lb force/50g sample)
water	34.6 ± 4.1[a]	17.4 ± 1.1[a]	43.3 ± 2.1[a]
12% AB	18.1 ± 0.8[b]	5.9 ± 1.4[b]	21.7 ± 3.1[b]
1:1	15.0 ± 1.7[b]	5.3 ± 0.9[b]	31.8 ± 1.0[c]
1:3	14.2 ± 2.7[b]	4.5 ± 1.8[b]	24.5 ± 2.3[bc]
1:7	18.5 ± 1.7[b]	6.9 ± 0.3[b]	22.5 ± 2.2[bc]
1:15	28.3 ± 3.2[a]	6.6 ± 2.7[b]	27.2 ± 1.6[bc]

[a-c] Means (± SD). Values in same column with same superscript not significantly different ($p \leq 0.05$)

Table 2—Sensory evaluation of cod fillets treated with 1:15 ammonium bicarbonate:sodium bicarbonate, saturated sodium chloride (NaCl) or distilled water (DW)

Attribute	Storage time (days)	Treatment DW	1:15	NaCl
Appearance	0	7.6 ± 2.9	8.7 ± 3.4	8.3 ± 3.2
	3	9.3 ± 2.8[a]	7.5 ± 3.5[b]	9.0 ± 3.1[a]
Aroma	0	8.2 ± 3.1[a]	6.8 ± 3.1[b]	8.2 ± 2.8[a]
	3	8.8 ± 2.8[a]	3.7 ± 2.6[b]	7.8 ± 2.8[a]
Flavor	0	8.6 ± 2.8	8.3 ± 2.4	8.1 ± 2.8
	3	7.5 ± 3.6[a]	5.6 ± 3.3[b]	6.6 ± 3.0[a]
Moisture	0	9.8 ± 2.7	9.3 ± 2.1	10.3 ± 2.4
	3	8.1 ± 2.3	9.4 ± 2.5	8.5 ± 2.3
Overall acceptability	0	8.7 ± 2.8	8.7 ± 2.5	8.5 ± 3.2
	3	7.6 ± 3.3[a]	5.1 ± 3.3[b]	7.4 ± 2.9[a]

[a-b] Means (±SD). Values in same row with no superscript or same superscript not significantly different ($p \leq 0.05$).

microbial load which was less than one log cycle below the 10^6 spoilage criterion at time of purchase (Figure 2). Cod dipped in solutions of various ratios of saturated AB to SB did however, exhibit delayed spoilage rates. At day 4, the saturated solutions of AB and 1:1 mixtures were most inhibitory (Fig. 2). The 1:3 and 1:7 solutions were slightly less effective. The 1:15 mixture, while least effective, still caused a one log reduction in total aerobic plate count. Untreated fish reached 10^6 cells/gram after one day while all treated fish reached this level about the fourth day.

After five days of refrigerated storage, treated cod were flakier than untreated controls and had significantly ($p<0.05$) improved texture by all three objective measurements (Table 1). Cooked drip was significantly less ($p<0.05$) for all treated samples except 1:15 AB:SB. On average, moisture loss was less than half that of untreated control. Centrifugal drip gave quantitatively similar results; bicarbonate treatments reduced drip loss by 50%. In those measurements, even the 1:15 treatment resulted in a statistically significant ($p<0.05$) decrease in moisture loss. Clearly, the treated samples retained moisture better than the untreated controls. This reduction of drip loss may, of itself, be an important new advantage of bicarbonates. Instron texture measurements of all treated samples were significantly ($p<0.05$) lower than those of untreated controls. The texture of cooked fish is closely related to its pH; that is, usually the lower the pH, the tougher the texture. pH apparently exerts its effect on the texture of fish muscle by influencing the contractile elements. A higher pH would promote unfolding and dissociation of proteins causing improvement in protein solubility and dispersibility and generally improving protein water absorption capacity (Hultin, 1985). All bicarbonate treatments increased pH of the fish from 7.0 to 8.2–8.5.

Results of the sensory evaluation indicated that, in its present form, bicarbonate dipping led to a product with inferior sensory characteristics (Table 2). After three days refrigerated storage the aroma, appearance, flavor, and overall acceptability of the 1:15 treated fish declined relative to the other samples. Note, however, that these differences developed over time. When evaluated on day 0, fish treated with the 1:15 mixture compared favorably with other samples in appearance, flavor, moistness and overall acceptability. Only the aroma of the 1:15 sample received a lower rating.

The sensory scores of bicarbonate dipped baked cod were significantly lower than the untreated fish after 3 days storage. However, we believe the improvement of microbial stability and decreased cook drip are of sufficient economic importance to warrant further investigation of bicarbonate dips in better controlled experiments where pre-experimental treatment of fish can be controlled from time of catch. The low aroma scores of the 3-day fish were due primarily to ammonia odor. This had not been observed in preliminary experiments where fish had been prepared by boiling (unpublished observation). In that case the water probably served to dilute the soluble ammonia. Future studies in our laboratory will focus on shrimp. Because they are commonly prepared by boiling, the ammonia odor problem should be reduced. Reduction of drip loss would be of major economic benefit to the shrimp industry.

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Biochemical Properties of Actomyosin of Cold Stored Striated Adductor Muscles of *Aulacomya ater ater* (Molina)

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ABSTRACT

Expressible juice showed highest increase within the second day of storage. Actomyosin was partially purified from stored muscles. Both reduced viscosity and Mg^{2+}-ATPase activity of actomyosin decreased about 44% in the first day. These changes were due neither to actomyosin dissociation nor fragmentation of major proteins of the complex. Relative percentage of myosin decreased, and actin increased in actomyosin after the second day. These changes are related to a decrease in the myosin heavy chain and could explain the slow decrease in reduced viscosity and Mg^{2+}-ATPase of this protein after the second day.

INTRODUCTION

THERE ARE MANY reports on the biological and biochemical composition of the bivalve *Aulacomya ater ater* (Molina), (Griffiths and King, 1979 a, b; Zaixo et al., 1979; De Vido de Mattio, 1983a). Seasonal variations in the biochemical composition of this mollusc were also reported (De Vido de Mattio, 1983b). Like other molluscs *Aulacomya* has two kinds of adductors, the larger of them contains striated muscle, and the smaller, smooth muscle. Extensive studies on the regulatory mechanism of both scallop muscles were made (Kondo et al., 1979; Chantler and Szent-Györgyi, 1980). Biochemical studies of the proteins constituting these muscles were also reported (Kondo and Morita, 1981). However, there are few reports on the biochemical properties of myofibrillar protein of *Aulacomya* (Milstein, 1967). Also, studies of effects of cold storage of adductor muscles on biochemical properties are lacking.

Visits to shellfish processing plants verify that adductor muscles lose liquid during cold storage up to freezing and frozen storage. Liquid loss in shucked oyster (*Crassostrea virginica*) meats during ice storage was reported (Cook et al., 1988). The great economic problem of weight loss during storage, freezing and thawing, or cooking of meat, is related to binding of water within the muscle. Myofibrillar proteins are responsible for the water holding capacity (WHC) and the other technological properties of the meat, such as emulsifying capacity and tenderness (Goll et al., 1977). The purpose of our work was to investigate the WHC and biochemical properties of actomyosin of striated adductor muscles stored at 2–4°C.

MATERIALS & METHODS

SPECIMENS of *Aulacomya ater ater* (Molina) were collected from February 1988 through January 1989 from SanJosé Gulf, Chubut, Argentina. Mature specimens 60–70 mm length were selected. Maturity of gonads was determined by macroscopic observation, and histology of the mantle by the procedure formerly described (Vinuesa and Tortorelli, 1980).

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After cleaning the shells, striated muscles were dissected. Muscles were carefully freed from adhering pancreatic and liver tissues, rinsed with 5 mM phosphate buffer (pH 7.0) containing 40 mM NaCl and 0.1 mM phenylmethylsulfonyl fluoride. Adductor muscles were stored up to 9 days at 2–4°C. Two experiments were performed. In each of them ten subsamples of eight adductor muscles were analyzed at zero time and 2, 5, 9 days of storage.

Actomyosin was obtained from adductor muscles by the modified method of Focant and Huriaux (1976). At zero time and at different storage periods, each subsample was homogenized in a Sorvall Omnimixer at full speed 30 sec in 30–40 vol cold 5 mM phosphate buffer containing 40 mM NaCl, 1 mM β-mercaptoethanol and 0.1 mM ethylene glycol bis (β-amino ethylether)N-N′ tetraacetic acid (EGTA). The homogenate was centrifugated at 10,000 × *g* 5 min, and the sedimented residue resuspended manually in the same solution, washed and centrifuged twice. The washed residue was resuspended at 5–10 mg protein per mL in the same solution, and the final solution had 0.6M NaCl and 5 mM ATP. The pH was maintained at 7.0 by addition of 0.5M Na_2HPO_4. After stirring 5 min, the solution was centrifuged as before for 20 min and the supernatant containing actomyosin filtered through gauze. Actomyosin was partially purified by the precipitation-solubilization method described by Tsuchiya et al., (1975). The pellet of crude actomyosin was solubilized in 20 mM Tris-maleate buffer (pH 6.8) containing 0.6M KCl, and aliquots were taken in order to perform Mg^{2+}-ATPase activity, reduced viscosity and ATP-response determinations. SDS-PAGE analysis of actomyosin was also performed.

Reduced viscosity

Reduced viscosity in actomyosin was measured at 20 ± 0.1°C using a Ubbelodhe viscosimeter by the procedure described by (Crupkin et al., 1979).

ATP-response

ATP-response of crude actomyosin was determined measuring the reduced viscosity before and after addition of ATP and Mg^{2+} up to 1 mM, and Ca^{2+} up to 0.1 mM (final concentration) under the following conditions: 0.6M KCl, 20 mM Tris-maleate buffer (pH 6.8) and 2 mg mL^{-1} of protein in 20 mL of total volume.

Protein determination

Protein concentration for actomyosin and myofibrillar protein extract was determined by the Lowry method, with bovine albumin as standard (Lowry et al., 1951).

Mg^{2+}-ATPase activity

Mg^{2+}-ATPase activity was measured in 0.15 mg mL^{-1} of actomyosin in a solution of Tris-maleate 20 mM (pH 6.8), 30 mM KCl, 2 mM $MgCl_2$, 1.6 mM ATP and 0.1 mM $CaCl_2$. The reaction was stopped after 1 min at 30°C with TCA at a 10% final concentration. Phosphorus was determined by the Chen method (Chen et al., 1956).

SDS-Polyacrilamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of actomyosin and myofibrillar protein extract was performed in 10% gels using a Shandon vertical gel apparatus, as previously reported (Portzio and Pearson, 1977).

Quantitative actomyosin composition was determined by scanning gels at 600 nm with a Beckman DU8 Spectrophotometer equipped with a gel scanning accessory. The protein loaded on the gel was

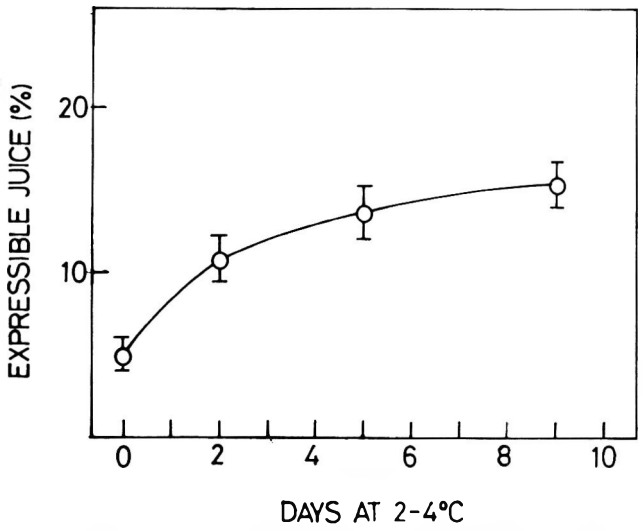

Fig. 1—Changes in expressible juice in cold stored muscles. Each point is mean of 8 determinations. Bars indicate confidence limits (P<0.05). Percent expressible juice is g expressible juice/100g muscle.

varied to check linearity of heavy myosin, actin and light myosin chain. With 30 μg of actomyosin, a linear response was obtained. Myosin/actin and myosin/paramyosin ratios were calculated by dividing heavy myosin plus myosin chain areas by actin and paramyosin respectively.

Expressible juice

Expressible juice was measured by Wierbicki et al., (1957) procedure with the modifications described by Ciarlo et al., (1985).

Statistical Analysis

Means were compared by analysis of variance and the Duncan's New Multiple Range Test (Steel and Torrie, 1960).

RESULTS & DISCUSSION

EXPRESSIBLE JUICE is a measurement of the water holding capacity (WHC) of the meat (Hamm, 1986). Changes in WHC are very sensitive indicators of the changes in the charges and structure of myofibrillar proteins (Hamm, 1960, 1975). Expressible juice reached the highest increase in cold stored muscles by the second day of storage (Fig. 1). These results suggest that modifications in both physicochemical and biochemical properties of myofibrillar proteins may occur during storage of adductor muscles. To investigate this possibility, both myofibrillar protein extract and crude actomyosin were obtained from striated adductor muscles.

SDS-PAGE gels of total myofibrillar protein extracts showed polypeptidic bands of the myosin heavy chain (MHC), paramyosin (PM), actin (A), tropomyosin (TM) and the myosin light chain (MLC) (Fig. 2). These bands were present in the SDS-PAGE gel of both myofibrils striated muscle of the scallop and muscle homogenate of *Mercenaria* (Elfvin et al., 1976; Kondo and Morita, 1981).

Tropomyosin is a tenacious contaminant of actomyosin purified from striated adductor muscle of scallop (Hashimoto and Watabe, 1976). However, TM is not essential for regulation of the molluscan muscle (Kendrick-Jones et al., 1970). Relative percentage of TM in crude actomyosin of *Aulacomya* was less than 2% (Fig. 2 and 4).

A polypeptidic band of 17 KDa was present in actomyosin gels. Regulatory and essential chains of scallop myosin comigrated on SDS containing gels (Szent-Györgyi et al., 1973; Kendrick-Jones et al., 1976). If a similar situation occured

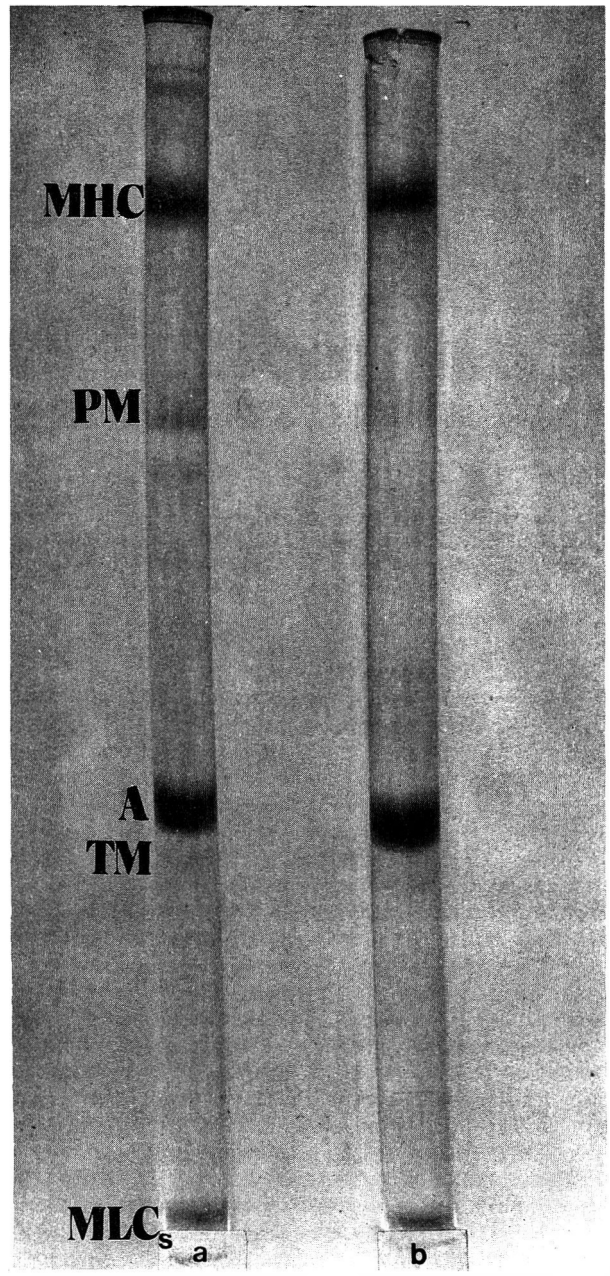

Fig. 2—SDS-PAGE 10% patterns of myofibrillar protein extract and crude actomyosin. MHC, myosin heavy chain, mol. wt. 200 KDa; PM, paramyosin, mol. wt. 110 KDa; A, actin, mol. wt. 42 KDa; TM, tropomyosin, mol. wt. 36 KDa; MLCs, myosin light chains, mol. wt. 17 KDa. a-Myofibrillar extract; b- Crude actomyosin.

with myosin light chains of *Aulacomya*, 17 KDa band could also correspond to both regulatory and essential myosin light chains. As judged by Mg^{2+}-ATPase activity and by Ca^{2+} dependence of the enzyme, both regulatory and essential chains were present in actomyosin preparation (Table 1).

Partially purified actomyosin had characteristic reduced viscosity, ATP-response and Mg^{2+}-ATPase activity (Table 1). About 44% of decrease occured in both reduced viscosity and Mg^{2+}-ATPase activity of crude actomyosin within the first day. Both parameters fell slowly thereafter up to the end of storage (Fig. 3).

Relative percentages of myosin, PM and actin in crude actomyosin at zero time were 50, 13 and 37% respectively (Table 2). A PM/M weight ratio higher than 0.3 had inhibitory effect on actin activated ATPase of myosin (Szent-Györgyi et al., 1971). Paramyosin stain 1.1 times more intensely with coom-

Table 1—Biochemicl and physicochemical properties of actomyosin

Protein	Reduced viscosity	ATP response	Mg^{2+} ATPase activity (μmol of Pi/min per mg protein) Pca 8[a]	6	5	4	3	2
Actomyosin	5.2 ± 0.7[b]	65 ± 5.0[b]	0.028	0.29	0.38	0.85	0.88	0.82

[a] Mg^{2+} ATPase activity at 10^{-8} M Ca^{2+} determined in presence of 0.5 × 10^{-4} M ethyleneglycol bis- (β aminoethylether) N-N'-tetraacetic acid (EGTA).
[b] Mean of six determinations ± confidence limits (P<0.05)

Table 2—Relative percentages of myosin, paramyosin, and actin; myosin/actin and myosin/paramyosin ratios in crude actomyosin from stored muscles

Days at 2–4°C	Relative percentage (%)[a] M	PM	A	Ratio[a] M/A	M/PM
0	50.1±4.2[b]	13.5±2.5[b]	36.8±4.9[b]	1.4±0.3[b]	3.8±0.6[b]
2	49.5±5.0[b]	14.5±2.4[b]	36.0±5.6[b]	1.4±0.4[b]	3.5±0.7[b]
5	40.2±4.0[c]	14.4±3.4[c]	45.1±3.2[c]	0.9±0.1[c]	3.0±0.8[c]
9	40.0±4.3[c]	14.7±2.0[c]	45.3±2.8[c]	0.9±0.1[c]	2.8±0.7[c]

[a] Mean ± confidence limits (n=6 P < 0.05). M = Myosin; PM = paramyosin; A = actin.
[b-c] Values in the same columns with different superscripts are significantly different (P < 0.01).

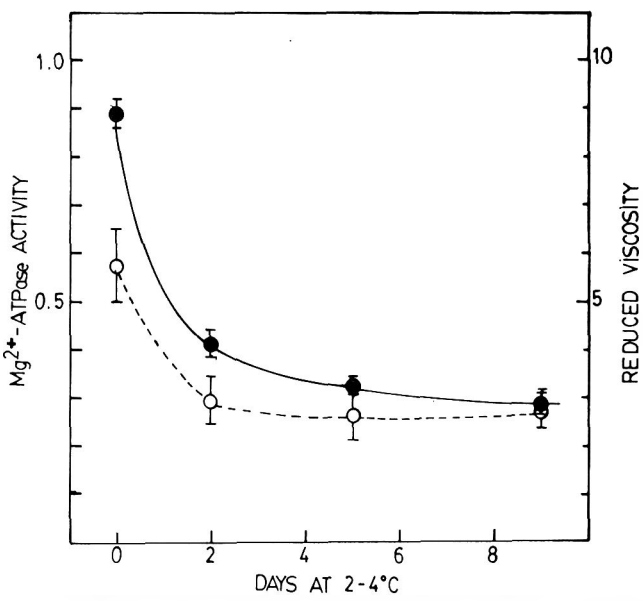

Fig. 3—Reduced viscosity and Mg^{2+}-ATPase activity of crude actomyosin from cold stored muscles: Reduced viscosity (○) and Mg^{2+}-ATPase activity (●). Mg^{2+}ATPase activities are micromoles of inorganic phosphate released within 1 min at 30°C per mg protein. Each point is mean of 12 determinations. Bars represent confidence limits (P<0.05).

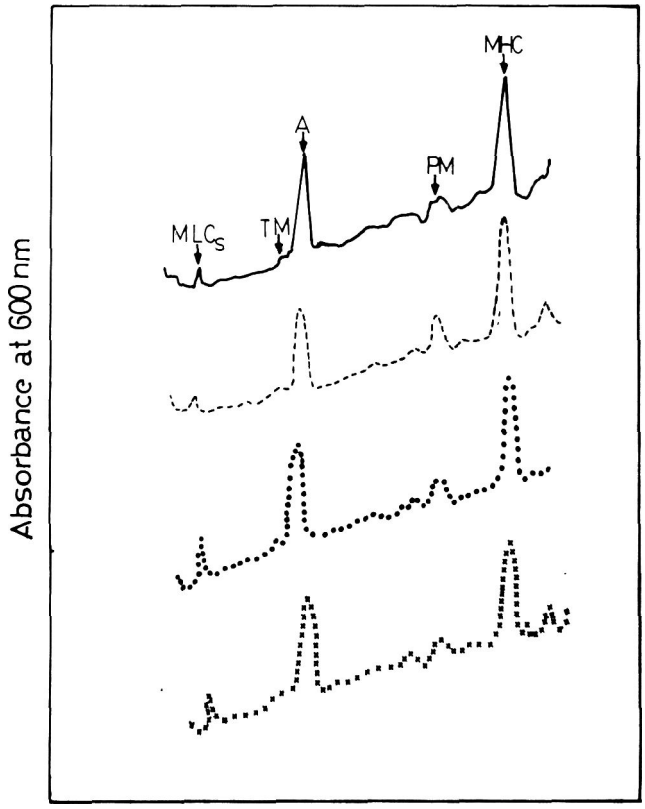

Fig. 4—Densitometric analysis profiles of SDS-PAGE gels of crude actomyosin from stored muscles: (———) 0 day, (---) 2 days, (···) 5 days, (xxx) 9 days. MHC, myosin heavy chain; PM, paramyosin; A, actin, TM, tropomyosin; MLC_s, myosin light chains.

assie brilliant blue than MHC (Levine et al., 1976). Taking into account this consideration, the PM/M weight ratio in actomyosin obtained from *Aulacomya* was about 0.2.

The relative percentage of myosin and actin in actomyosin was unchanged up to the second day of storage (Table 2). Relative percentage of myosin significantly decreased and that of actin likewise increased thereafter up to the end of storage. Relative percentage of paramyosin in actomyosin of cold stored striated muscles was unchanged.

A significant decrease in myosin/actin ratio and a trend to decrease in the myosin/paramyosin ratio after the second day of storage were observed (Table 2). Results of densitometric analysis of gels after SDS-PAGE 10% of actomyosin obtained from cold stored muscles are shown in Fig. 4. The decrease of the MHC area in actomyosin obtained from cold stored muscles are shown in Fig. 4. The decrease of the MHC area in actomyosin gels started on the second day of storage. Though the nature of changes which led to less MHC in actomyosin of cold stored muscles is not known, changes in both viscosity and Mg^{2+}-ATPase activity within the second day are not due to that.

It is widely accepted that modifications in the Mg^{2+}-ATPase activity occur when actin-myosin interaction changes are produced. The changes in both Mg^{2+}-ATPase activity and reduced viscosity within the first day could be explained by a decrease in myosin-actin interaction which led to actomyosin dissociation. However, this possibility is not supported by the results of Table 2, because if myosin and actin dissociate from the actomyosin complex of stored adductor muscle, a decrease in relative percentages of both and an increase in relative percentage of paramyosin would be expected. Conformational changes in actomyosin at the catalytic site of the enzyme could be involved although further investigations are necessary to demonstrate such. MHC decrease could be related to the slow fall in reduced viscosity and Mg^{2+}-ATPase activity after the second day.

In our work it was demonstrated that the greatest changes in both WHC and biochemical properties of myofibrillar pro-

tein occur in cold stored muscles by the second day of storage. Decrease in WHC results in decrease in tenderness and other quality factors of the meat (Hamm, 1975). Tenderness is highly related to the state of myofibrillar proteins (Marsh et al., 1974). Thus, changes in tenderness and other quality factors of the meat could be expected in cold stored *Aulacomya* muscles. Changes in both flavour and taste in ice stored raw shucked scallop have been reported (Hardy and Smith, 1970).

CONCLUSIONS

ACTOMYOSIN of *Aulacomya* denaturalized during cold storage of striated adductor muscles. This change in the major myofibrillar protein was accompanied by a decrease in WHC of the meat.

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Influence of Enzymatic Hydrolysis on Structure and Emulsifying Properties of Sardine (*Sardina pilchardus*) Protein Hydrolysates

G.B. QUAGLIA and E. ORBAN

ABSTRACT

The influence of enzymatic modification with Alcalase was studied on surface hydrophobicity, molecular weight distribution and emulsifying properties of ten hydrolysates from sardine differing in degree of hydrolysis. Surface hydrophobicity was evaluated fluorometrically using 1 anilino-8-naphthalenesulfonate (ANS); molecular weight distribution was determined by gel chromatography on a Superfine Sephadex G50. Emulsifying capacity and stability were evaluated as functions of pH values and product concentration, in comparison with sodium caseinate. Results showed emulsifying properties, surface hydrophobicity and the high molecular weight fraction decreased as degree of hydrolysis increased. Thus production of hydrolysates with desired molecular structures and emulsifying properties is possible.

INTRODUCTION

VARIOUS PROTEIN PRODUCTS are becoming available for food use (Hynd, 1970; Delaney, 1976; Kinsella, 1979; Satterlee, 1981; Smith and Brekke, 1984). Evaluation of their functional properties is necessary and helps food processors to select the right protein for a given application or to define how a protein or derivative can replace other proteins in food formulations. Emulsifying properties of proteins play an important role in many different food applications (Schweiger, 1974; Waggle et al. 1981; Evans, 1982 Parks and Carpenter, 1987) and are among the more important properties which need to be evaluated. Various factors and conditions influence emulsifying properties of proteins or their derivatives. These include: chemical and structural characteristics i.e. the content and arrangement of amino acid, molecular size, hydrophobic regions and conditions (temperature, pH and ion effects) prevailing in the medium (Kinsella, 1979; Parker, 1987). In this context, the purpose of our work was to study the influence of different degrees of hydrolysis on some structural parameters (surface hydrophobicity, molecular weight distribution) and emulsifying capacity and stability of ten hydrolysates, obtained by controlled enzymatic hydrolysis of sardine (*Sardina pilchardus*) with Alcalase.

MATERIALS & METHODS

Enzyme

Alcalase 0.6 Anson μg^{-1} 0.6 from Novo Industry.

Substrate

Comminuted and defatted sardines (*Sardina pilchardus*) were used as substrate. Sardines were eviscerated and washed with water, comminuted in a meat grinder and defatted by extracting with isopropanol (1/1 v/w) 3 times at 46°C for 30 min each. The residue was homogenized in water (1/4) with a blender and subjected to enzymic hydrolysis in pH stat, @pH8.5, 60°C. After the required digestion time, the mixture was heated to 90°C 2 min and centrifuged at 4000 rpm 7 min. The soluble phase was decanted and spray-dried in a laboratory dryer with inlet 170°C and outlet 85°C. Samples were stored as hydrolyzed sardine protein powder.

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Preparation of protein samples

Ten products differing in degree of hydrolysis were prepared. The hydrolyses were performed in a pH-stat at optimum pH and temperature conditions for Alcalase. The controlling parameter of the process was degree of hydrolysis (DH). Those selected were: 5%, 10%, 15%, 20%, obtained by varying either enzyme/substrate ratio (from 0.1 to 2%) or hydrolysis times. The times were drawn from hydrolysis curves where DH was plotted against time at constant pH and temperature for different enzyme/substrate ratios. Such curves were obtained previously in a study aimed at determining hydrolytic capacity of the enzyme on sardine protein (Quaglia and Orban, 1987).

Determination of emulsifying properties

Emulsifying capacity was determined by an oil titration method (Webb et al., 1970). This method is suitable for less viscous emulsions. Hydrolysates were tested at concentrations of 1%, except for those with DH 5% that were tested at 1%, 2% and 4% concentration (between 400 mg/50 mL and 1.6 g/50 mL of protein concentration) in 1.0M NaCl and distilled water in a pH range 1–8. Sodium caseinate was compared with the hydrolysates. The process was carried out in a glass vessel connected with a refrigerated water bath, to keep the temperature of the emulsion at 20°C during processing. The mixtures were emulsified at 10,000 rpm with a homogenizer (Super Dispergerate, PBI International), maize oil was continuously added from a 500 mL separatory funnel. A volt-ohmeter (Kyoritsu Electrical Instrument Works LTD) was used to monitor increase in electrical resistance which occurred upon emulsion collapse. Since the protein content of the products and sodium caseinate were not identical, their weights were adjusted such that the protein concentration was the same for all samples evaluated. Results were expressed as mL oil emulsified for each protein concentration.

Emulsion stability test. To determine stability of all emulsions, the apparatus, speed of mixing and rate of oil addition were identical to those used in determining emulsifying capacity, but in this case the oil addition was stopped 10 mL before the previously determined end point. A stability test which determines extent of moisture homogeneity between initial and tested samples of the emulsion was used. The method is a modification of that introduced by Titus et al. (1968), the measurement of change in moisture rather than oil and the lengthening of the stability testing period from 2 to 24 hr were included. Two 25 mL samples of each emulsion were placed in 22x150 mm test tubes. The two samples were held at 4°C for 2 hr and then placed in a 37°C incubator 24 hr. Two additional 5 mL aliquots of the emulsion were immediately analyzed for moisture following the AOAC (1965) procedure. Following 37°C incubation the bottom 5 mL of the samples were removed with a 5 mL pipette and analyzed for percent moisture. A stability rating for each sample was determined on the basis of percent change in moisture. The following equation was used:

$$SR = \frac{100 - M_{test}}{100 - M_{original}} \times 100$$

where SR is the stability rating; M_{test} the percent moisture of the bottom 5 mL of the sample stored at 37°C for 24 hr, and $M_{original}$ is the initial percent moisture of the sample.

Table 1—Milliliters of emulsion oil, as function of pH, from hydrolysates with different degrees of hydrolysis (DH) prepared with different enzyme/substrate ratios (E/S). Solvent 1M NaCl. Product concentration of the solutions 1%, protein concentration 400 mg/50 mL.

Products		pH						
E/S%	DH%	2	3	4	5	6	7	8
0.1	5	165	150	130	140	165	160	135
0.5	5	185	170	120	115	160	140	135
1	5	160	150	125	125	160	145	130
2	5	165	150	130	140	140	160	150
0.5	10	130	120	115	120	125	130	120
1	10	130	115	105	110	115	120	110
2	10	120	115	105	110	120	125	135
1	15	115	105	90	95	100	95	100
2	15	115	110	90	95	100	100	95
2	20	100	90	85	85	95	80	80

Table 2—Stability ratings, as function of pH, of oil-in-water emulsions stabilized by protein hydrolysates with different degree of hydrolysis (DH) prepared with different enzyme/substrate ratio (E/S). Solvent 1M NaCl. Product concentration of the solutions 1%. Protein concentration 400 mg/50 ml.

Products			pH						
E/S%	DH%	Hours	2	3	4	5	6	7	8
0.1	5	2	98.4	98.4	87.9	69.5	64.5	61.9	51.5
		24	90.2	63.3	61.5	56.5	52.5	53.8	—
0.5	5	2	97.6	98.5	88.1	79.4	58.5	41.4	41.3
		24	93.6	64.7	55.6	37.1	28.6	20.0	—
1	5	2	96.5	98.9	82.5	66.2	48.0	31.6	28.3
		24	89.6	56.6	51.6	22.3	—	—	—
2	5	2	95.0	99.4	63.5	36.0	21.5	23.2	15.5
		24	92.4	57.6	63.9	—	—	—	—
0.5	10	2	40.2	41.2	28.5	26.0	27.0	19.5	16.5
1	10	2	41.2	40.6	24.3	25.1	24.5	20.3	13.2
2	10	2	40.1	41.3	21.6	22.2	10.6	—	—

Molecular characterization of the hydrolysates

The molecular weight distribution of the ten products was carried out by gel chromatography on a Superfine Sephadex G 50, in a column (1.5x110 cm), with a bed volume of approximately 260 mL. The effluent flow was controlled by a peristaltic pump and the effluent was monitored at 280 nm by a UV detector connected to a recorder. The eluent was distilled water. Bovine albumin (MW 67,000), chymotrypsinogen (MW 25,000), myoglobin (MW 17,800), cytochrome C (MW 12,300) trypsin inhibitor (MW 6,500) and Bacitracin (MW 1,450) were used as standard proteins.

Surface hydrophobicity, was determined by the method of Kato and Nakai (1980) measuring fluorescence resulting from binding of 1-anilinonaphthalene 8-sulphonate (ANS) to the hydrophobic regions using a Perkin Elmer LS 5 Fluorescence Spectrometer. Protein extracts were diluted to 0.020% protein using 0.01M phosphate buffer pH 7 and 0.01 citrate buffer pH 5. ANS-protein conjugates were excited at 390 nm and the relative fluorescence intensity values (RFI) were measured at emission wavelength 486 nm. RFI was adjusted to 30 in 100 full scale for the standard consisting of ANS in methanol. Protein blank RFI values were measured for each protein dilution in the absence of ANS. The initial slope was calculated from the fluorescence intensity vs protein concentration.

RESULTS & DISCUSSION

THE LOWER the degree (e.g. 5%) of hydrolysis the samples had undergone, the higher was their emulsifying capacity, itself a function of pH, as evidenced by hydrolysates at 1% concentration (400 mg/50 mL of protein concentration) dispersed in 1M NaCl (Table 1). At pH < 4 and > 5 the emulsifying capacity (EC) of the protein lysates (except for those at 15% and 20% degree of hydrolysis) increased. The EC in 15% and 20% samples showed a linear trend above pH 4. Low (DH 5%) hydrolysis products were more susceptible to the different pH values of the medium. Hydrolysates dispersed in water showed no significant difference with those dispersed in NaCl. Where they were used as the emulsifying agent (Table 2), those that had undergone a lower degree of hydrolysis (DH 5%) revealed a capacity for stabilising emulsions at both 2 hr and 24 hr, this capacity being higher in the pH 2–4 range but less as pH increased. We studied products that had undergone 5% degree of hydrolysis in comparison of their emulsifying capacity with the relative stability of emulsions obtained with sodium caseinate, which is widely used as an emulsifer in the food industry. Figure 1 illustrates the emulsifying capacity of a hydrolysate prepared with an enzyme/substrate ratio of 0.1% of all products, at 5% degree of hydolysis. Whether in water or in NaCl the hydrolysate had higher emulsifying capacity than sodium caseinate throughout the pH range except at pH 8 where findings were identical for the two. Increasing ion concentration had a negative effect on sodium caseinate, particularly in an acid medium (pH 2–4). As for the stability of these emulsions (Fig. 2), in the pH 6–8 range, both in water and in 1M NaCl and at pH 2–3 in water, sodium caseinate stabilized emulsions better than hydrolysates. In an acid me-

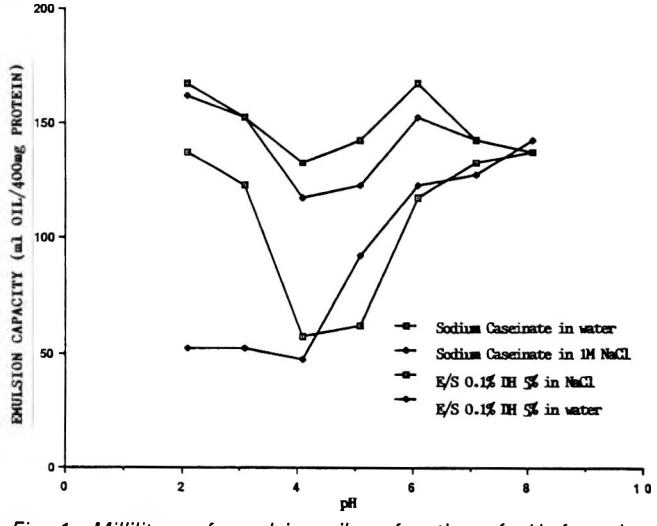

Fig. 1—Milliliters of emulsion oil as function of pH, from hydrolysates with degree of hydrolysis (DH) 5% prepared by using enzyme/substrate ratio (E/S) 0.1% and sodium caseinate both in water and 1M NaCl. Product concentration of the solution 1%, protein concentration 400 mg/50 mL.

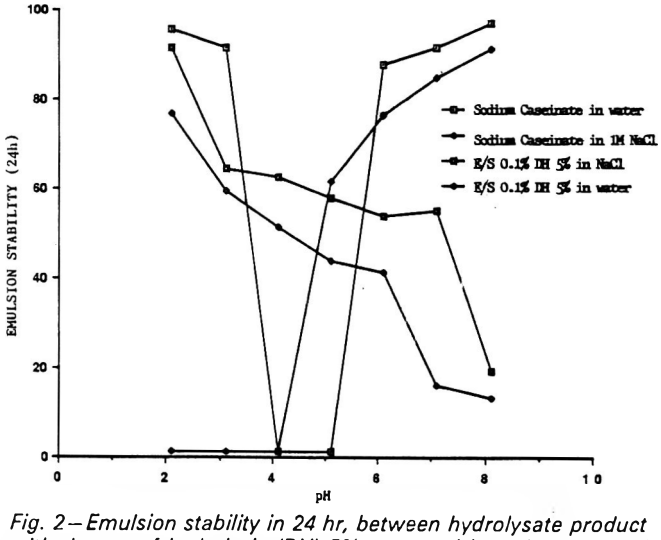

Fig. 2—Emulsion stability in 24 hr, between hydrolysate product with degree of hydrolysis (DH) 5% prepared by using enzyme/substrate ratio (E/S) 0.1% and sodium caseinate both in water and in 1M NaCl. Product concentration of the solution 1% in water and in 1M NaCl.

Table 3—Effect of hydrolysate concentration increase on mL of oil in emulsion as function of pH, from the hydrolysates with degree of hydrolysis (DH) 5% prepared with different enzyme/substrate ratios (E/S). Solvent 1M NaCl.

Products E/S%	Conc %	pH 2	3	4	5	6	7	8
0.1	1	165	150	130	140	165	160	135
	2	170	160	145	150	160	155	155
	4	180	160	125	145	180	160	150
0.5	1	185	170	120	115	160	140	135
	2	185	170	130	130	165	155	160
	4	180	165	125	130	190	140	150
1	1	160	150	125	125	160	155	145
	2	165	155	140	135	180	170	150
	4	170	160	120	135	165	165	160
2	1	165	150	130	140	140	160	150
	2	165	160	145	150	145	180	145
	4	170	160	115	135	160	160	155

Table 4—Effect of hydrolysate concentration increase on stability ratings (24 hr) of emulsions stabilized by protein hydrolysates with degree of hydrolysis (DH) 5% prepared with different enzyme/substrate ratios. Solvent 1M NaCl.

Products E/S%	Conc. %	pH 2	3	4	5	6	7	8
0.1	1	90.2	63.3	61.5	56.6	52.5	53.8	–
	2	98.9	99.2	98.6	87.1	60.0	52.4	–
	4	98.7	98.1	96.8	98.0	96.9	94.4	65.0
0.5	1	93.6	64.7	55.6	37.1	28.6	20.0	–
	2	95.2	94.4	90.3	87.7	49.0	52.7	–
	4	97.5	98.3	98.0	98.8	95.0	89.0	65.0
1	1	89.6	56.6	51.6	22.3	–	–	–
	2	93.8	90.5	89.7	78.1	43.5	48.6	–
	4	97.8	96.9	97.7	98.1	96.3	87.5	58.4
2	1	92.4	57.6	63.9	–	–	–	–
	2	92.0	88.7	85.9	60.2	–	–	–
	4	95.4	96.2	97.7	95.1	64.4	78.4	48.0

Table 5—Surface hydrophobicity S_o for products with different N degree of hydrolysis (DH).

Products E/S (%)	DH (%)	Hydrophobicity (S_o) pH 7	pH 5
2	20	800	1087
2	15	862	1098
1	15	877	1112
2	10	887	1112
1	10	885	1137
0.5	10	925	1200
2	5	937	1212
1	5	937	1212
0.5	5	1012	1712
0.1	5	1825	1975

dium, however (pH 5–2), in 1M NaCl, caseinate had no further stabilizing capacity. A further test was carried out on low-hydrolysis (DH 5%) products for the effect of increasing concentration of hydrolysate from 1% to 4%, on emulsifying capacity and the relative stability over 24 hr. An increase in concentration of the hydrolysate (Table 3) had no significant effect on the emulsifying capacity, whereas the stability of the emulsion increased considerably over 24 hr. As Table 4 shows, a 4% concentration corresponded to a very high stability, throughout the pH range for all emulsions produced with lysates obtained from 5% hydrolysis. Similar evidence is afforded by a further test where the emulsions were left at room temperature for 4 days, with no change.

Surface hydrophobicity (S_o) and molecular weight composition

As indicated in Table 5 showing results of S_o determination for all hydrolysates, with different degrees of hydrolysis the surface hydrophobicity increased as extent of hydrolysis decreased. This result was the same both at pH 7 and pH 5. From the molecular weight distribution profile (Fig. 3) for the hydrolysates with lowest (DH 5%) and highest (DH 20%) hydrolysis, in the range of molecular weights 30,000 to 1000, a decrease of the high molecular weight fraction was noted as degree of hydrolysis increased. The profile of molecular weight distribution for the products with intermediate degree hydrolysis (DH 10%; DH 15%) was similar to the sample with DH 20%.

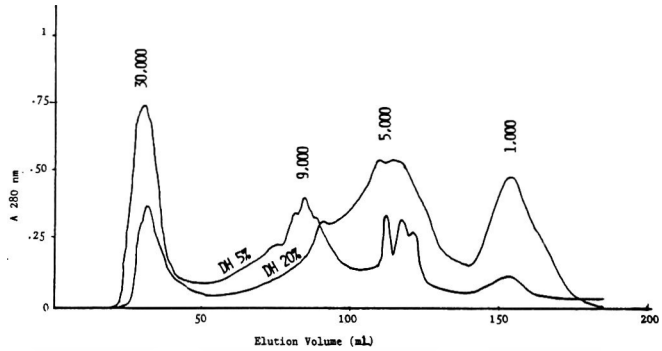

Fig. 3—Molecular weight distribution for the hydrolysate with degree of hydrolysis (DH) 5% prepared by using enzyme/substrate ratio (E/S) 0.1% and the hydrolysate with degree of hydrolysis (DH) 20%. Absorbance 280 nm.

These results indicated enzymic hydrolysis had a negative influence on hydrolysates capacity to form and stabilize emulsions as degree of hydrolysis increased. Hydrolysates with low degree hydrolysis had emulsifying properties higher than the commercial product employed for this purpose, sodium caseinate. As previously shown, products with lower degrees of hydrolysis (DH 5%) had higher surface hydrophobicity than the other hydrolysates. This supports the hypothesis that effective hydrophobicity plays an important positive role in determining emulsifying properties (Kato and Nakai, 1980; Nakai 1980; Voutsinas et al., 1983). The different molecular weight distribution of the hydrolysates, derived from degrees of hydrolysis, showed that the higher content of high protein fraction seemed to play an important role in the emulsifying properties, especially in stabilizing the emulsion.

In conclusion hydrolysis with proteolytic enzymes provides the possibility of controlling cleavage degree of protein in the substrate. Using suitable enzyme/substrate ratios and times, this permits production of hydrolysates with different molecular structures and different emulsifying properties that could find application in various food applications.

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Color Evaluation in Raw, Baked and Smoked Flesh of Rainbow Trout (*Onchorhynchus mykiss*) Fed Astaxanthin or Canthaxanthin

GRETE SKREDE, TROND STOREBAKKEN and TORMOD NÆS

ABSTRACT

Color of rainbow trout (*Onchorynchus mykiss*) was investigated using sensory and instrumental analysis. When judged in pairs with equal carotenoid concentration, astaxanthin caused less whiteness, higher chromaticity and more red hue of trout flesh than canthaxanthin. Sensory assessed whiteness, chromaticity and red hue significantly correlated with instrumental values for lightness, chromaticity and hue [CIE (1976) L*a*b* and $H(^0)_{ab}$]. Multivariate regression analysis improved the predictability for all sensory variables compared with univariate analysis. Prediction of carotenoid concentration from instrumental values was better for astaxanthin than for canthaxanthin.

INTRODUCTION

THE PIGMENTS of salmonid flesh are carotenoids. The pigments are absorbed from the diet and distributed to the fish muscles where they bind to actomyosin (Henmi et al., 1987). The most abundant carotenoid for wild salmonides, astaxanthin, and canthaxanthin have both been used in diets for farmed fish (Francis and Clydesdale, 1975; Little et al., 1979; Choubert, 1982; Storebakken et al., 1986; Gullestad, 1988; Skrede et al., 1990; Torrissen et al., 1989). The two pigment types have slightly different light absorption properties and cause differences in flesh color of Atlantic salmon (*Salmo salar*, L.) (Skrede and Storebakken, 1986a).

Color of salmonid flesh may be assessed by various analytical methods; sensory analysis using trained panelists for descriptive or comparative tests (Ostrander et al., 1976; Skrede and Storebakken, 1986a) or comparison of fish samples with standardized colors (Francis and Clydesdale, 1975; McCallum et al., 1987; Skrede et al. 1990); by instrumental analysis based on light reflected from flesh samples (Francis and Clydesdale, 1975, Little et al., 1979; Skrede and Storebakken, 1986a, b) or by quantitative carotenoid analysis of salmonid flesh (Schiedt et al., 1981; Yamazaki et al., 1983; Torrissen et al., 1989). When results from various analytical methods are to be compared, difficulties may arise because of lack of intensity references in sensory analysis or lack of standardized colors for comparison. Instrument design and sample presentation will influence values from instrumental color analysis (Skrede and Storebakken, 1986b; Baardseth et al., 1988) and in pigment analysis the choice of extraction and calculation procedures may influence results (Skrede and Storebakken, 1986a). For practical purposes, knowledge of results of chemical or instrumental methods and how they relate to perceived color of salmonid flesh, is important (Little et al., 1979, Skrede and Storebakken, 1987).

Our investigation was undertaken to study how pigmentation with astaxanthin or canthaxanthin influence raw and processed flesh color as determined by sensory analysis and by instrumental color and pigment analysis of farmed rainbow trout (*Onchorynchus mykiss*). A further aim was to investigate relationships between sensory and instrumental methods to see how instrumental methods can be used to evaluate perceived trout flesh color.

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MATERIALS AND METHODS

Fish material

Rainbow trout (*Onchorynchus mykiss*) were raised in net pens in the sea and fed 25, 50 or 100 mg/kg diet of either astaxanthin (Carophyll Pink, 5%) or canthaxanthin (Carophyll Red, 10%, F. Hoffmann-La Roche Ltd, Basle, Switzerland) as described by Bjerkeng et al. (1990). Two control groups received no carotenoids in the feed. During 16 wk feeding the body weight increased from about 500g to 1 kg. After slaughtering, the trout were stored up to 2 months at −20°C until analysis.

Ten rainbow trout fed astaxanthin and 10 fed canthaxanthin were included in the experiments. The trout were selected by analysis according to their carotenoid content to cover the widest possible range in carotenoid concentration obtained through feeding. The trout were further selected to make the fish match, each pair consisting of trout with equal concentrations of astaxanthin and canthaxanthin. In addition, one pair was selected from the control groups which received no carotenoids. This pair was selected for low carotenoid content of flesh. The carotenoids extracted from trout fed astaxanthin were: 95–99% astaxanthin, 5–1% canthaxanthin. The carotenoid composition of the trout fed canthaxanthin was: 94–99% canthaxanthin, 0–1.5% astaxanthin, 0–2.6% carotenes, and 1–2% unidentified carotenoids. A pooled sample of the flesh from the control group contained 50.2% astaxanthin and 49.8% canthaxanthin (Bjerkeng et al., 1990). Carotenoid analyses were performed with flesh cut posterior to the adipose fin. Two slices (9 mm thick) cut anterior to the adipose fin were used for color analysis of raw trout. The remaining fish body was filleted. One fillet from each fish was baked, the other was smoked. Baking and smoking methods were described previously (Skrede and Storebakken, 1986a).

Carotenoid analysis

Carotenoid concentrations of trout were determined by acetone extraction (Skrede and Storebakken, 1986a). Absorbance was read at 475 nm and an extinction coefficient $E^{1\%}_{1cm}$ of 1900 was used to calculate both astaxanthin and canthaxanthin.

Sensory analysis

Visual assessment of color was performed with a 12 member sensory panel. Panelists were trained to evaluate color according to the NCS system (SSI, 1979) where color variables are defined as whiteness/blackness, chromaticness and hue. In our study the NCS term chromaticness is designated chromaticity. The evaluations were performed in individual booths with 500 lux daylight fluorescent lighting on the tabletop (Colorette/Luma, Sweden). The trout samples were presented to panelists as flesh plugs cut by a cork borer (diam 22 mm, ht 9 mm). Samples were viewed with a grey background (L* = 80.7, a* = − 0.7, b* = 0.6). Each trout was analyzed twice in random serving order.

Trout were analyzed individually using intensity scales (1–9) for whiteness, blackness, chromaticity and yellow and red hue, (Amerine et al., 1965). Increasing intensity of each parameter was given increasing values. The results for each sample were reported as the average of the scores. Trout were also evaluated in a paired test where fish containing astaxanthin were tested against fish containing about the same concentration of canthaxanthin (Amerine et al., 1965). The panelists were asked to evaluate whiteness, chromaticity and red hue

Table 1—Ranges for carotenoid concentration of raw flesh and CIE L, a*, b* and $H(^0)_{ab}$ values of raw, baked and smoked flesh of rainbow trout pigmented with astaxanthin or canthaxanthin*

	Astaxanthin	Canthaxanthin
Carotenoid conc., mg/kg	6.4 – 15.0	6.4 – 14.4
Raw trout		
Lightness, L*	43.5 – 49.0	45.7 – 52.9
Redness, a*	9.1 – 14.7	4.3 – 8.0
Yellowness, b*	15.5 – 23.4	15.6 – 22.5
Hue, $H(^0)_{ab}$	57.9 – 61.6	67.2 – 74.5
Baked trout		
Lightness, L*	66.8 – 71.4	70.3 – 72.4
Redness, a*	6.2 – 11.0	3.5 – 4.8
Yellowness, b*	15.6 – 20.8	15.9 – 19.6
Hue, $H(^0)_{ab}$	61.0 – 69.2	75.5 – 78.9
Smoked trout		
Lightness, L*	38.2 – 42.7	41.9 – 45.2
Redness, a*	6.6 – 12.0	4.0 – 6.7
Yellowness, b*	8.7 – 15.8	8.7 – 14.3
Hue, $H(^0)_{ab}$	50.6 – 57.5	63.4 – 69.1

Table 2—Carotenoid concentration and significance levels for differences in whiteness, chromaticity and red hue in pairs of raw rainbow trout fed astaxanthin or canthaxanthin as assessed by sensory analysis. Instrumentally obtained differences in chromaticity (ΔC) and hue ($\Delta H(^0)_{ab}$) are given

	Carotenoid conc. mg/kg		Sensory color[a]			Instrumental color	
Pair no.	Astaxanthin	Canthaxanthin	Whiteness	Chromaticity	Red hue	ΔC	$\Delta H(^0)_{ab}$
1	3.4	6.4	ns	ns	***	3.4	10.4
2	8.6	8.4	**	***	***	5.9	9.3
3	8.8	8.7	ns	***	***	4.2	8.1
4	9.8	9.5	ns	*	***	5.0	11.3
5	9.5	9.3	***	***	***	10.2	14.8
6	10.1	10.3	ns	***	***	9.0	11.3
7	10.1	10.2	**	**	***	7.4	8.8
8	12.4	12.2	ns	***	***	6.3	12.3
9	12.3	12.3	***	***	***	5.2	11.6
10	15.0	14.4	***	***	***	9.4	13.0
$\bar{x}$						6.6	11.1
s						2.3	2.0

[a]Significance levels: ***P<0.001; **P<0.01; *P<0.05.

within each pair and to select the sample which had the most pronounced level of each color.

Instrumental color analysis

Instrumental color analysis was performed with a Minolta Chroma Meter II CR-100 reflectance instrument. The instrument had an 8 mm diam measuring area. Prior to analysis, the instrument was calibrated with a white standard. Light source C was used throughout the experiment. Samples were measured in a cell (Minolta CR-A50) with a black metallic bottom, white sides from a Teflon ring and a glass top through which measurements were taken. The diameter of samples, to fit into the measuring cell, was 22 mm, height 9 mm. Color variables calculated by the instrument are CIE L*a*b* adopted in 1976 of which L* describes lightness, a* red-green chromaticity, and b* yellow-blue chromaticity. Hue $H(^0)_{ab}$, values were calculated as $\tan^{-1} = b^*/a^*$ ($H(^0)_{ab} = 0$ for red, $H(^0)_{ab} = 90$ for yellow) (Hunter and Harold, 1987). The instrumental analyses were performed on the trout samples used for sensory judgment just after sensory analysis. The glass top of the measuring cell decreased CIE readings by 2.4 for L*, 1.2 for a* and 0.4 for b*. Color values of trout samples were not corrected for these.

Statistical analysis

The relationship between color variables of the sensory tests and instrumental color variables were investigated by linear regression methods both in univariate and multivariate modes (Weisberg, 1985).

The univariate methods were based on the model

$$y = \beta_o + \beta x + e \quad (1)$$

where y is the score for a sensory variable and x is one of the instrumental variables CIE L*, a*, b*, $H(^0)_{ab}$ or the carotenoid concentrations. The multivariate method was based on the model

$$y = \beta_o + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + e \quad (2)$$

where y again is sensory score and x_1, x_2, x_3, x_4 are the variables L*, a*, b* and $H(^0)_{ab}$, respectively. The β's in the model are the regression coefficients to be determined, while e is the error term representing lack of fit of data to the model plus random error. Regression coefficients were computed by the least squares criterion (Weisberg, 1985). For each univariate regression, the correlation between y and x, as well as the ordinary estimate of the standard deviation of e, $\hat{\sigma}$, were computed. For the multivariate regressions, the correlation between measured sensory score (y) and that predicted from the calculated regression (y), was computed. These correlations were to be compared with the absolute value of correlation between y and x for the corresponding univariate regressions. In addition, the standard deviation of e, σ, was estimated as in the univariate case. While the correlation coefficient r measures the error of model fit relative to the variability of the variables. $\hat{\sigma}$ measures the absolute error.

In principle, the regressions should be evaluated on new samples not involved in the initial analysis. To test the validity of the regressions a cross-validation (Stone, 1974) was performed. This corresponded to dividing data into subsets and calculating regressions the number of times that there are subsets, each time leaving one of the subsets out of the analysis. For each of these calculations, the regression equation was tested on the subset not involved in the calibration. For all correlations raw, baked and smoked trout were treated separately.

RESULTS

Fish material

The ranges for carotenoid concentration of raw flesh and the instrumentally obtained color values of raw, baked and smoked flesh of the rainbow trout pigmented with astaxanthin or canthaxanthin are reported in Table 1. The pigment ranges for the two types of carotenoid were similar. Despite this, differences were found in instrumental color values for flesh pigmented with the two types of carotenoid. As shown by the ranges for color values in Table 1 astaxanthin caused lower L* values, higher a* values and lower $H(^0)_{ab}$ values than canthaxanthin in raw, baked and smoked flesh. Only b* values were similar in flesh from trout fed the two types of pigments. For both astaxanthin- and canthaxanthin-pigmented trout, color values changed during processing (Table 1). Baking increased lightness and decreased redness. Thus, the baked flesh had a more yellowish hue than the raw flesh. Upon smoking, the flesh turned darker, decreased in redness and yellowness, but obtained in a more reddish hue compared with raw flesh.

Comparing color of astaxanthin- and canthaxanthin-pigmented trout by sensory and instrumental analysis

When raw trout flesh pigmented with astaxanthin was compared with flesh pigmented with equal amounts of canthaxanthin by paired sensory tests, significant differences in whiteness and chromaticity were found in most pairs (Table 2). Visually judged differences in hue were significant for all pairs. Panelists judged trout with astaxanthin less white and having higher chromaticity and a more red hue than trout with canthaxanthin. Also for baked and smoked flesh significant differences were obtained within most pairs (results not presented). Exceptions were whiteness and chromaticity in baked and smoked flesh of the pair with the lowest pigment concentration (no 1) and whiteness in baked flesh of the pair with the highest pigment concentration (no 10) where no differences were detectable.

From instrumental color analysis, the pairs of raw flesh judged as significantly different in chromaticity by sensory analysis, had chromaticity differences, ΔC, above 3.5 (Table 2). Differences in instrumental hue values ($\Delta H(^0)_{ab}$) within pairs all exceeded 8.0. For baked and smoked flesh, ranges for chromaticity differences within pairs were 3.0–6.8 and 3.9–8.9, respectively (data not shown). No lower limit for chromaticity

Table 3—Correlation coefficients from univariate regression analysis between sensory (y) and instrumental (x) variables describing color of raw, baked and smoked trout flesh containing astaxanthin or canthaxanthin

Sensory variable y	Instrumental variable x	Correlation coefficient[a] Raw	Baked	Smoked
Whiteness	Lightness, L*	0.71	0.83	0.84
Blackness	Lightness, L*	0.12	0.54	0.08
Chromaticity	Redness, a*	0.93	0.95	0.94
Chromaticity	Yellowness, b*	0.81	0.66	0.75
Hue, red	Hue, $H(^0)_{ab}$	−0.94	−0.95	−0.87
Hue, yellow	Hue, $H(^0)_{ab}$	0.47	0.36	0.49

[a] Significance level $P<0.001$ for correlation coefficients >0.65; $P<0.01$ for coefficient >0.54.

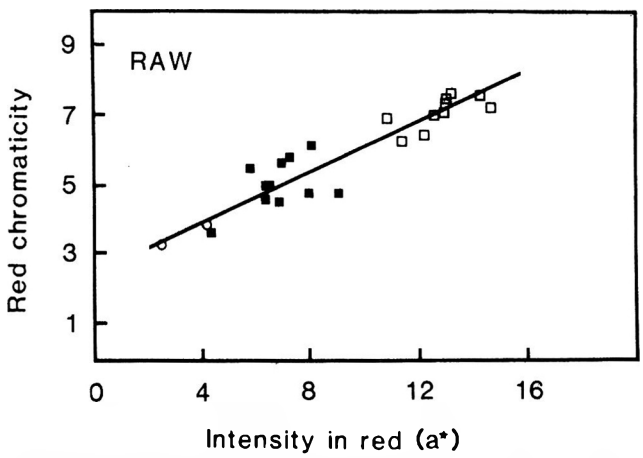

Fig. 1—Red chromaticity assessed by sensory analysis of trout fed no pigment (○), astaxanthin (□) or canthaxanthin (■) versus intensity in red (CIE a) obtained by instrumental analysis. Univariate regression (r=0.93): chrom = 2.70 + 0.340 (a*). Multivariate regression (r=0.96): chrom = 5.23 − 0.153 (L*) + 0.357 (a*) + 0.025 (b*) + 0.064 $(H(^0)_{ab})$.*

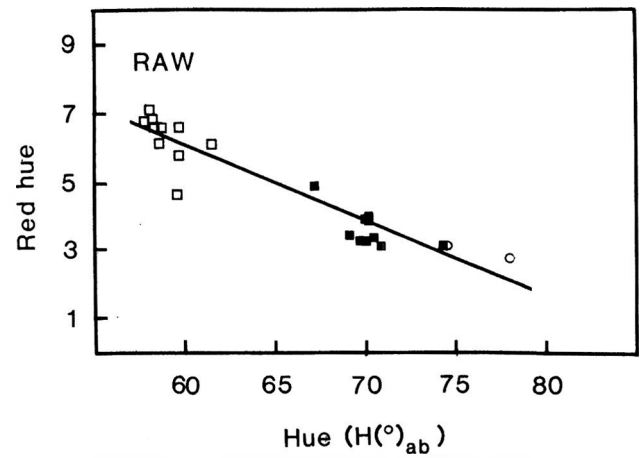

Fig. 2—Red hue assessed by sensory analysis of trout fed no pigment (○), astaxanthin (□) or canthaxanthin (■) versus hue (CIE $H(^0)_{ab}$) obtained by instrumental analysis. Univariate regression (r=−0.94): Red hue = 13.45 − 0.134 $(H(^0)_{ab})$. Multivariate regression (r=0.98): Red hue = −1.36 − 0.055 (L) + 0.741 (a*) − 0.249 (b*) + 0.105 $(H(^0)_{ab})$*

differences corresponded to the limit of visual differentiation between the two types of carotenoids. Average differences in chromaticity (ΔC) and in hue ($\Delta H(^0)_{ab}$) within pairs as calculated from the instrumental analysis, were not significantly different in raw, baked and smoked trout flesh.

Table 4—Correlation coefficients between measured (y) and predicted (ŷ) sensory scores from the multivariate regression analysis based on CIE L, a*, b* and $H(^0)_{ab}$ values of raw, baked and smoked trout flesh containing astaxanthin or canthaxanthin*

Sensory variable	Correlation coefficient[a] Raw	Baked	Smoked
Whiteness	0.81	0.93	0.94
Blackness	0.82	0.73	0.87
Chromaticity	0.96	0.96	0.96
Hue, red	0.98	0.97	0.95
Hue, yellow	0.93	0.94	0.87

[a] Significance level $P<0.01$ for correlation coefficients >0.75.

Table 5—Estimate of $\hat{\sigma}$ (standard deviation of the random error in the regression model) from univariate regression analysis based on sensory score and CIE L, a*, b* and $H(^0)_{ab}$ values of raw, baked and smoked trout flesh containing astaxanthin or canthaxanthin*

Sensory variable y	Instrumental variable x	$\hat{\sigma}$ Raw	$\hat{\sigma}$ Baked	$\hat{\sigma}$ Smoked
Whiteness	Lightness, L*	0.78	0.66	0.67
Blackness	Lightness, L*	0.63	0.42	0.69
Chromaticity	Redness, a*	0.50	0.47	0.41
Chromaticity	Yellowness, b*	0.79	1.19	0.86
Hue, red	Hue, $H(^0)_{ab}$	0.53	0.51	0.79
Hue, yellow	Hue, $H(^0)_{ab}$	0.80	0.87	0.95

Relationships between sensory and instrumentally assessed color

Correlation coefficients from the univariate regression analyses between sensory and instrumentally assessed color of trout containing astaxanthin and canthaxanthin, are presented in Table 3. For raw, baked and smoked trout flesh correlations were significant for sensory assessed whiteness, chromaticity and red hue with the instrumental values for lightness (L*), chromaticity (a*, b*) and hue ($H(^0)_{ab}$), respectively. Best relationships between sensory and instrumental analysis of raw flesh were those of red chromaticity (Fig. 1) and hue (Fig. 2). Sensory assessed blackness and yellow hue correlated less well with corresponding instrumental variables.

Results demonstrated that the instrumental values for color of trout flesh are a good measure for visually perceived color regardless of whether the flesh is pigmented with astaxanthin or canthaxanthin (Fig. 1 and 2). Similar graphs were obtained for baked and smoked trout flesh (not presented). To optimize prediction of sensory assessed color from instrumental color values, multivariate regression analysis was applied in addition to univariate analysis already presented.

When, in multivariate regression analysis, each sensory variable was tested toward combined instrumental variables (Table 4), correlation coefficients were directly comparable with absolute value of those from the univariate tests (Table 3). For all combinations tested, correlation coefficients were higher in the multivariate mode than in the univariate. Especially for sensory assessed blackness and yellow hue, correlation coefficients increased when four, instead of one, instrumental color parameters were included.

Estimated standard deviation, $\hat{\sigma}$, of the error term e in model (1) or (2) is a measure of fit of the regression model to the data. Low values for $\hat{\sigma}$ correspond to better fits than higher values. In the univariate case (Table 5), the estimated standard deviations, $\hat{\sigma}$, in raw and baked trout were lowest for the regressions involving sensory assessed red chromaticity and hue. For smoked trout only chromaticity-redness demonstrated similar values for the estimated standard deviation. Thus, univariate linear regression analysis, was better suited for studying correlations involving sensory assessed red chromaticity and hue than those involving whiteness, blackness and yellow chromaticity and hue.

With multivariate regression analysis (Table 6), the differences in estimated standard deviations, $\hat{\sigma}$, for various sensory

Table 6—Estimate of σ (standard deviation of the random error in the regression model) from multivariate regression analysis based on sensory score and CIE L, a*, b* and $H(^0)_{ab}$ values of raw, baked and smoked trout flesh containing astaxanthin or canthaxanthin*

Sensory variable y	$\hat{\sigma}$ Raw	$\hat{\sigma}$ Baked	$\hat{\sigma}$ Smoked
Whiteness	0.57	0.47	0.48
Blackness	0.49	0.37	0.37
Chromaticity	0.41	0.48	0.41
Hue, red	0.36	0.48	0.55
Hue, yellow	0.36	0.36	0.59

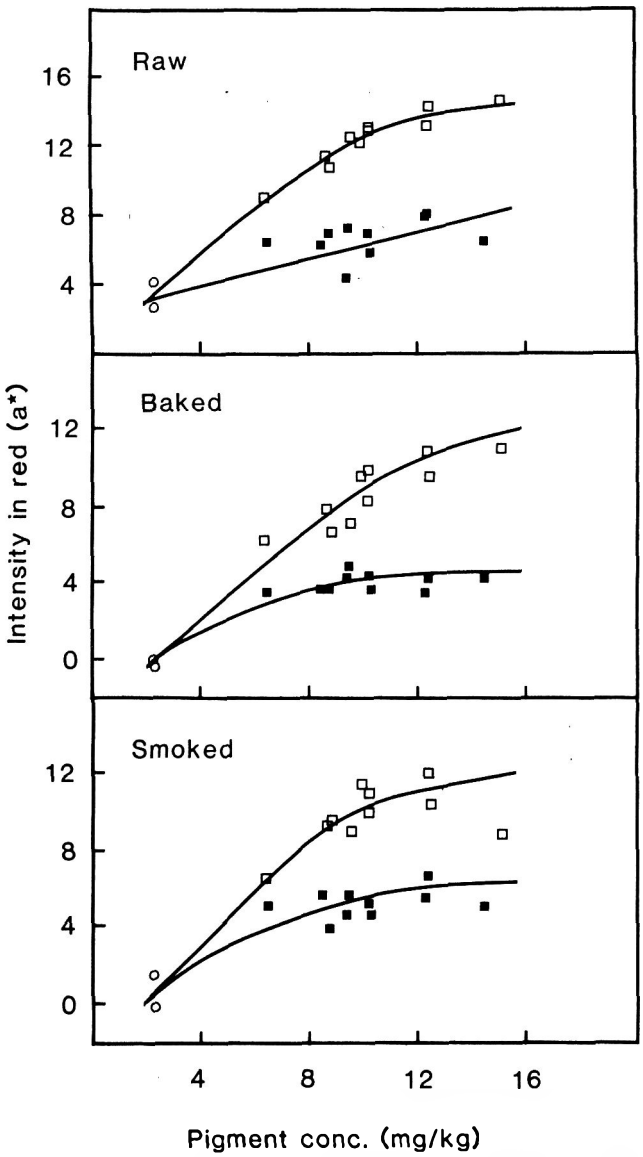

Fig. 3—Instrumentally obtained intensity in red (CIE a) versus pigment concentration of raw, baked and smoked trout fed no pigment (○), astaxanthin (□) or canthaxanthin (■).*

variables were lower than with univariate analysis. In general, $\hat{\sigma}$ was lower in the multivariate mode than in the univariate, ranging from 0.4 to 0.6 units. This indicates better fits of linear regressions when all instrumental variables were included. The cross-validation gave similar results as the statistical fitting results measured by $\hat{\sigma}$, thus supporting the validity of the findings.

The results thus clearly demonstrated that linear relationships existed between sensory and instrumental color analysis, and were best described by using multivariate regression analysis compared with univariate analysis. With multivariate analysis, sensory score could be predicted with a standard error of prediction of about 0.5.

Table 7—Correlation coefficients[a] between color values and carotenoid concentration of raw, baked and smoked flesh of rainbow trout

	Astaxanthin	Canthaxanthin
Raw trout		
Lightness, L*	−0.94	−0.40
Redness, a*	0.96	0.76
Yellowness, b*	0.93	0.74
Hue, $H(^0)_{ab}$	−0.87	−0.64
Baked trout		
Lightness, L*	−0.80	−0.63
Redness, a*	0.95	0.84
Yellowness, b*	0.92	0.82
Hue, $H(^0)_{ab}$	−0.90	−0.80
Smoked trout		
Lightness, L*	−0.55	−0.34
Redness, a*	0.85	0.82
Yellowness, b*	0.62	0.58
Hue, $H(^0)_{ab}$	−0.84	−0.79

[a] Significance level $P<0.001$ with correlation coefficients >0.84.

Relationships between instrumentally assessed color and carotenoid concentration

Plots of redness of raw, baked and smoked flesh (Fig. 3) vs carotenoid concentration of raw flesh demonstrated a significant difference in instrumental response to carotenoid concentrations, between the two pigment types. Astaxanthin caused much higher color values of the flesh than canthaxanthin at equal pigment concentrations.

With astaxanthin, redness increased nearly linearly with increasing pigment. When canthaxanthin was used, redness to a much lesser extent depended on pigment concentration. These differences were found in raw, baked and smoked flesh. The results thereby demonstrated that if instrumental color analysis is to be used for predicting pigment concentration, each pigment type should be analyzed separately.

As a consequence of the differences between the two types of pigment, the correlation coefficients between CIE color values of raw, baked and smoked flesh and pigment concentration for raw flesh were calculated for each pigment separately (Table 7). For both pigments there was a slight but significant, indication that best correlations were between redness (a*) and carotenoid concentration. However, the use of linear regression for canthaxanthin-pigmented trout within the concentration range of our experiment, is disputable.

DISCUSSION

THE CONCENTRATION RANGE for astaxanthin of rainbow trout of our study (6.4–15.0 mg/kg), was wider than previously reported. Seurman et al. (1978) reported astaxanthin concentration of raw flesh between 0.4 and 1.6 mg/kg after feeding red crab to rainbow trout. Similarly, Chen et al. (1984) reported from 5.2 to 9.8 mg astaxanthin/kg after feeding with astaxanthin-enriched soy oil extracts from crawfish. Choubert and Luquet (1983), using shrimp meal in the diet, obtained astaxanthin concentrations of 1.4 mg/kg dry matter. With a dry matter content of 26% (Skrede and Storebakken 1986a) this would correspond to 5.4 mg astaxanthin per kg trout flesh. For canthaxanthin, Storebakken et al. (1986) reported concentrations from 8.8 to 19.0 mg/kg in rainbow trout fed synthetic canthaxanthin. This corresponds well with the levels obtained in our experiment.

In our study, the sensory panelists were trained in evaluating whiteness/blackness, chromaticity and red/yellow hue of the trout color separately. By this splitting of visual color into separate dimensions, close relationships were found between color evaluation by sensory analysis and that by instrumental analysis. Statistical analysis revealed whiteness, blackness,

yellow hue and chromaticity were better predicted from instrumental variables by multivariate than by univariate methods. However, when predicting chromaticity from CIE a* values and red hue from $H(^{\circ})_{ab}$ values, differences between the two were minor. Schmidt and Idler (1958) and Saito (1969) previously reported instrumental values for redness served to describe differences in red obtained by visual evaluation of salmon flesh. Little et al. (1979), however, previously reported the necessity of combining all CIE L*a*b* parameters by multiple regression analysis to describe sensory evaluated color. This is in agreement with our finding, although univariate analysis may be sufficient to evaluate color intensity and hue for practical purposes.

Processing caused changes in visually perceived salmonid flesh color. This has also been reported previously; cooking resulted in a shift in hue from red to pinkish, a lighter and less pure color (Chen et al., 1984) and a lighter color with less purity and hue shift from red to orange-red (Francis and Clydesdale, 1975) for astaxanthin-pigmented trout and salmon flesh, respectively. Instrumental color of raw, baked and smoked trout flesh was related to the carotenoid concentration of the raw flesh. With the close relationships demonstrated between instrumental and sensory color in our study, this conclusion is also valid for visual color of trout flesh. The relationship between visual and instrumental color descriptions and pigment concentration was better for astaxanthin than for canthaxanthin.

We concluded that color of rainbow trout, as with Atlantic salmon, depends upon type and concentration of pigment in the flesh. Color differences between astaxanthin and canthaxanthin pigmented trout were demonstrated in raw, baked and smoked flesh. Highly significant relationships between sensory perceived color and instrumentally assessed color were found. Multivariate regression analysis revealed more valid relationships than univariate analysis. For raw, baked and smoked trout flesh pigmented with astaxanthin, instrumental color analysis can be used to predict pigment concentration. In trout with canthaxanthin, instrumental color analysis proved less suitable as color values remained nearly constant with increasing pigment concentration.

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Lipid Composition and Flavor Changes in Irradiated Mango (var.Alphonso)

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ABSTRACT

Changes were studied in fatty acid composition of the pulp, aroma and flavor of gamma irradiated (0.25kGy) and control mature green Alphonso mangoes during ripening at 25–30°C. Ripening of both control and irradiated mangoes was accompanied by changes in glycerides as well as fatty acids. Oleic acid of pulp oil of irradiated mangoes decreased appreciably during ripening as compared with controls. Linoleic acid of pulp oil of unirradiated fruits decreased markedly on the 6th day of storage. With irradiated fruits linoleic acid remained unaffected up to the 12th day of storage. Linolenic acid content of pulp oil of irradiated fruit increased much more than that of unirradiated fruit during ripening. Gas chromatographic profiles of volatiles of control and irradiated mangoes showed no difference.

INTRODUCTION

GAMMA IRRADIATION at low levels improved the shelf-life of tropical fruits such as mangoes, banana and papayas by delaying the process of ripening and senescence. In studies with mangoes, variety Alphonso (a popular commercially important variety in India), Mathur and Lewis (1961) were first to report improvement in shelf life by irradiation. Dharkar and co-workers (1966) showed Alphonso mango treated with 0.25 kGy of gamma irradiation had extended shelf-life by about 6-8 days at ambient temperature. Many research workers (Ahmed et al., 1973; Dennison and Ahmed, 1967; Thomas and Beyers, 1979) used irradiation as a tool for extending shelf-life of mango varieties. They investigated effects of gamma irradiation on physicochemical properties like ascorbic acid and sugar content, acidity, total carotenoids during storage, and reported no significant change in these quality attributes except delay in ripening. Blakesley et al. (1979) also reported no changes in volatile components, amino acids or fatty acids in Kent mango irradiated to 0.75 kGy. However, few reports are available on changes in the important constituents with respect to flavor genesis such as lipid and volatile components in gamma irradiated mango during ripening. Lipids contribute as precursors to aroma constituents of several fruits (Tressl and Drawert, 1977; Salunkhe and Do, 1976). Bandyopadhyay and Gholap (1973) in their study on changes in fatty acids of ripening mangoes at ambient temperature observed the ratio of palmitic : palmitoleic acid correlated with aroma and flavor characteristics of mangoes. The purpose of our investigation was to assess lipid composition and its role in aroma and flavor characteristics of gamma irradiated Alphonso mango during post harvest storage.

MATERIALS & METHODS

Materials

Two hundred mangoes Var. Alphonso were procured from a local market in green-mature unripe state. They were divided into two groups, each with 100 fruit. One group was subjected to gamma-irradiation and the other kept as unirradiated control. Each group was then subdivided into four independent lots and the pulp of five mangoes from each lot was taken at different ripening stages for subsequent analysis.

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Irradiation

The fresh green mangoes were exposed to 0.25 kGy (optimum dose for this variety) of gamma radiation from Cobalt 60 gamma cell 220 (Atomic Energy of Canada Ltd.) dose rate 0.09 kGy/min measured by Fricke dosimetry. Irradiated mangoes along with unirradiated control were allowed to ripen in a well-ventilated room at 25–30°C.

Fruits were selected at different stages of ripening for analysis. The stages of ripening were determined by a panel of six expert judges and were graded as follows: Unripe—peel green and pulp hard and sour without ripe aroma; Half-ripe—peel yellowish green and pulp slightly soft with perceptible ripe aroma; Table-ripe—peel uniformly yellow, pulp soft and sweet with characteristic ripe aroma and flavor; Fully-ripe—peel orange-yellow, pulp soft and sweet with characteristic ripe aroma and flavor; Over-ripe—peel dark orange, peel shrunken and tender, pulp very sweet with less aroma.

Methods

Preparation of mango pulp. Fruits were peeled and sliced after rejecting the seed. Slices were homogenized to uniform pulp using a Waring Blendor. All operations were carried out at 0°C. Fresh pulp was used for isolation of aroma concentrate.

Isolation of aroma. Pulp of irradiated (15 days stored), and unirradiated (12 days stored) table ripe mangoes was used for isolation of aroma volatiles. The procedure detailed elsewhere (Bandyopadhyay et al., 1973) was essentially based on isolation of volatile components by high vacuum distillation followed by extraction of odorous principles from distillate with diethyl ether and finally distillation of ether at low temperature under high vacuum. These concentrates, subjected to organoleptic test, represented the true aroma of the fruit.

Analysis of aroma. The aroma concentrates were analyzed by gas liquid chromatography (GLC) using a Shimadzu GC 4A gas chromatograph, equipped with a thermal conductivity detector and a dual column of stainless steel (3 mm diam × 3m) packed with 20% ethylene glycol succinate on acid washed Chromosorb P (60-80 mesh). Column was maintained at 130°C, while the injection port and detector were at 150°C. The carrier gas was helium at a flow rate of 30 mL/min. Each of the GLC separated components of mango essence was subjected to descriptive odor test at the column exit by a panel of three experienced judges and the characteristic odor note of each was recorded on the chromatogram. The odor notes green mango-like, fruity, intense fruity, soily, almond-like, burnt sugar-like, sweet sugary and coconut oil-like of GC separated components of mango essence reported earlier (Bandyopadhyay et al., 1973) were identical to those we obtained from irradiated fruit. The aroma concentrate of ripe mango was further studied by GC-Mass spectrometry (GC-MS) combination at the U.S. Army Natick RD&E Center (Natick, MA). The GC was a modified Barber Coleman Model 5060 equipped with a 2% Carbowax 20 M and Versanimide 900 wall coated open tubular stainless steel column (0.03 in. diam × 500 ft). Helium was carrier gas at 6 mL/min. The column was programmed from 70° to 190°C at 5°C/min, after which it was held at 190°C until completion of the separations. The effluent of the capillary column was connected directly into the ion source of a fast scanning quadrupole mass spectrometer. The ionizing voltage was 70 eV and source was 200°C. Major components were identified by comparing the mass spectra of authentic substances reported in the literature.

Pulp oil extracts. The pulp oil of all samples was isolated by extracting the pulp with cold peroxide-free diethyl ether in a Waring Blendor and the amount was determined gravimetrically according to the procedure of Gholap et al., 1971.

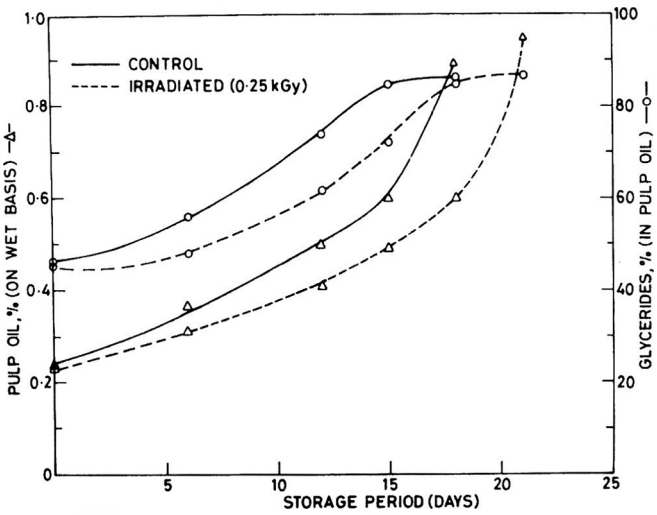

Fig. 1—Changes in pulp oil (△) and glycerides (○) of pulp from unirradiated (———) and irradiated (----------) Alphonso mango.

Analysis of pulp oil. The fatty acid composition of pulp oil of all samples was determined by GLC as methyl esters by the conventional procedure consisting of saponification followed by acidification and finally methylation by diazomethane (Bandyopadhyay and Gholap 1973). The GLC analysis of fatty acid methyl esters of each sample was carried out with a Bhabha Atomic Research Centre model GC equipped with a flame ionization detector. A 0.25 in.o.d. × 6 ft stainless steel column packed with 20% ethylene glycol succinate on 60-80 mesh chromosorb W(AW) was used. The oven and detector were maintained at 185°C with nitrogen flow of 25 mL/min. The fatty acids were identified by comparing retention times of authentic reference samples.

The glyceride content of each sample was estimated from a 0.1% chloroform solution of the extract by a colorimetric method using chromotropic acid (Borkenoogen, 1968).

RESULTS & DISCUSSION

MANGO, being a climacteric fruit, at post harvest ripening at ambient temperature is associated with a rapid change in color, flavor and texture after half-ripe state. Alphonso mango normally attained eating-ripe state on 10–12th day of storage with development of characteristic exotic aroma and flavor. The effect of gamma-irradiation at 0.25 kGy appeared to delay the overall ripening process without impairing detectable changes in physicochemical or biochemical parameters influencing quality of the fruit (Thomas, 1986). Gas chromatographic separation of the aroma concentrates of control and irradiated mangos indicated a close similarity in overall volatile profiles between the two. Over fifty compounds were separated by capillary GC in both cases and twenty-three major compounds were tentatively identified by GC-MS as follows: acetaldehyde, acetone, ethyl alcohol, 2-methyl propene, cisocimene, ethyl formate, methyl acetate, ethyl acetate, methyl vinyl ketone, N -butyl acetate, N-amyl acetate, α-pinene, β-pinene, dimethyl butane, N - undecane, dodecanol, benzaldehyde, benzonitrile, Γ-caprolactone, benzyl alcohol, Γ-octalactone, Γ-undecalactone and δ-octalactone.

The changes of pulp oil extracts and their glyceride contents at different ripening stages in control and irradiated fruits are summarized in Fig 1. A parallel increase in pulp oil and glyceride content of both control and irradiated fruit was evident. However, the rate of change was relatively slower in irradiated mangoes than in the controls. In case of unirradiated sample a maximum level of glyceride (85%) in pulp was attained on 15 days storage at fully ripe state. The irradiated sample exhibited the same level on 18 days storage at the same state of ripeness. Thus a delay of at least 3 days in ripening of mango was achieved by irradiation with 0.25 kGy.

The fatty acid composition of ether extracts of pulp from control and irradiated mangoes during ambient temperature storage is shown in Table 1. Changes in fatty acid composition of both samples followed similar patterns during ripening. An appreciable decrease in oleic acid of pulp oil of irradiated man-

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Table 1—Fatty acid composition (%wt) of glycerides from pulp of ripening, control and irradiated Alphonso mango stored at 25–30°C

	Days storage										
	Control					Irradiated					
	0	6	12	15	18	0	6	12	15	18	21
$C_{12:0}$	0.3	0.2	0.2	0.3	0.4	Trace	0.1	Trace	0.1	Trace	0.1
$C_{14:0}$	0.8	2.9	3.0	3.0	2.5	1.3	1.1	2.5	2.7	2.5	2.5
$C_{16:0}$	28.8	26.9	23.8	27.5	25.7	30.3	28.1	28.2	28.1	28.1	27.5
$C_{16:1}$	17.6	24.5	26.2	31.6	30.2	17.2	17.7	21.5	32.8	30.7	31.6
Unknown	Trace	0.3	0.8	0.7	0.7	–	Trace	1.4	1.4	2.2	1.8
$C_{18:0}$	1.3	1.3	1.2	2.4	1.3	1.3	1.1	Trace	1.4	1.6	2.3
$C_{18:1}$	25.2	25.8	24.1	18.9	22.2	27.0	29.5	23.1	10.9	11.4	11.5
$C_{18:2}$	15.7	4.3	2.8	1.4	1.5	12.8	12.2	11.7	1.2	1.3	1.2
$C_{18:3}$	10.3	13.6	17.9	14.2	15.5	10.1	10.2	21.6	21.4	22.2	21.5

Each value represents the average of four independent lots.

Table 2—Sensory evaluation with reference to aroma and flavor of control and irradiated mango stored at 25–30°C and correlation with ratios of palmitic to palmitoleic acid and oleic to linolenic acid

Samples	Days storage	Ripeness	Aroma[a]	Flavor[a]	$C_{16:0}/C_{16:1}$	$C_{18:1}/C_{18:3}$
Control	0[b]	Green-unripe	Nil	Nil	1.64	2.4
	6	Half-ripe	+	+	1.10	1.9
	12	Table-ripe	+ + +	+ + +	0.91	1.4
	15	Fully-ripe	+ + +	+ + +	0.87	1.3
	18	Over-ripe	+ +	+ +	0.85	1.4
Irradiated (0.25 kGy)	0	Green-unripe	Nil	Nil	1.70	2.7
	6	Green-unripe	Nil	Nil	1.59	2.9
	12	Half-ripe	+	+	1.31	1.1
	15	Table-ripe	+ + +	+ + +	0.86	0.5
	18	Fully-ripe	+ + +	+ + +	0.92	0.5
	21	Over-ripe	+ +	+ +	0.87	0.5

[a] Organoleptic score: +, very mild, + +, mild, + + +, strong
[b] Freshly picked, unripe mangoes

Delayed Light Emission as an Indicator of Peach Maturity

W. R. FORBUS, JR. and G. G. DULL

ABSTRACT

Delayed Light Emission as an Indicator of Peach Maturity. W. R. Forbus, Jr. and G. G. Dull. USDA-ARS, Richard B. Russell Agricultural Research Center, P. O. Box 5677, Athens, GA 30613. Keystone, Loring, and Nectar peaches were evaluated to determine relationships between delayed light emission (DLE) and physical and chemical properties that have been shown to be related to peach maturity. Results showed that DLE was highly correlated with average maturity rank ($r = 0.96$) for all three cultivars. DLE can provide an effective, nondestructive technique for measuring peach maturity.

INTRODUCTION

PEACHES and many other fruits are often produced long distances from the points where they are consumed. Efficient marketing dictates that such fruits be harvested before they are fully ripe, and changes that occur during ripening take place during shipment. Fruits that continue to ripen after they are harvested are said to be climacteric. Excessive losses occur in marketing these fruits because methods are not available for nondestructively measuring maturity at all stages of marketing.

Methods evaluated for estimating peach maturity include subjective comparison of ground color with color charts, measuring flesh firmness with a Magness-Taylor pressure tester, titratable acidity, and total chlorophyll (Rood, 1957). Deshpande and Salunkhe (1964) suggested the ratio of soluble solids to acids could serve as a reliable index to peach maturity and Sidwell et al. (1961) reported eating quality of Elberta peaches could be determined on the basis of chlorophyll. Other indices of physiological maturity proposed include total spectral reflectance (Bittner and Norris, 1968; Long and Webb, 1973), and ground color (Delewiche and Baumgardner, 1983; 1985). Color chips have been developed for assessing peach maturity in the orchard and are applicable to a wide range of cultivars (Delewiche and Baumgardner,1985). These color chips have been used by researchers to assess maturity for experimental purposes (Shewfelt et al., 1987; Meredith et al., 1989). These methods are either slow, subjective or require destruction of the sample. There is a need in the peach industry for a technique to rapidly and nondestructively measure maturity of peaches.

Since physiological maturity is related to chlorophyll content, the use of delayed light emission (DLE) measurements has potential as a means of nondestructively measuring peach maturity. DLE is a low intensity light emitted from chlorophyll-containing plant material for several seconds, or minutes after it has been illuminated with a light source (Strehler and Arnold, 1951). The intensity of the DLE produced was shown to depend on concentration of chlorophyll and related compounds in the product. In the dark the DLE can be detected with a light-sensitive detector such as a photomultiplier tube. The relationship between DLE and maturity has been established for tomatoes (Chuma and Nakaji, 1976; Forbus et al., 1985), satsuma oranges (Chuma et al., 1977), bananas (Chuma et al., 1980), papaya (Forbus et al., 1987; Forbus and Chan, 1989) and cantaloupe (Forbus and Senter, 1989).

Our study was conducted to evaluate the relationship between DLE and the physical and chemical properties of peaches known to be related to maturity. Results will provide a basis to evaluate feasibility of DLE as a nondestructive measure of peach maturity.

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MATERIALS & METHODS

Samples

Keystone, Nectar and Loring peaches were harvested from a local commercial orchard. Keystone and Loring cultivars are yellow-fleshed while fruit of the Nectar cultivar is white-fleshed. Based upon visual judgements of size and color, almost 100 fruits of similar size and representative of the full range of peach maturities from immature through tree ripe were harvested for each cultivar. It was necessary to harvest fruit on two different dates for each cultivar to obtain the full range of maturities. Thirty-five fruits of each cultivar were selected and used for physical and chemical analyses.

Physical measurements

DLE measurements were made on the surface of each fruit on the blossom end and on the blush and ground color areas using equipment and procedures described previously (Forbus and Chan, 1989). DLE values were recorded in volts of detector response from a 19.6 cm^2 area of fruit surface 0.25 sec after illumination.

Hunter color values were obtained using a Hunterlab D25-2 colorimeter equipped with a 5.08 cm diameter viewing port on the optical head. L(lightness), a (green to red), and b (yellow to blue) values were measured on the external surface of each fruit at the three positions where the DLE measurements were made. Hunter a and b values obtained from each position were used to compute values for hue angle ($\theta = \tan^{-1} b/a$), a parameter that has been shown effective for predicting visual color appearance (Little, 1975).

Firmness measurements were made on two opposite pared cheeks of each fruit, at positions 90° apart around the circumference and midway between the blossom and stem end, with a Magness-Taylor pressure tester having an 8 mm diameter tip. Firmness measurements at the two positions were averaged and expressed in Newtons.

Chemical measurements

Six 2.5 cm diameter cores, two from each position where DLE measurements were made, were removed from each peach. A core included the skin and tissue from the skin to the pit. A 3 mm thick disc was sliced from the skin end of each core. Discs were individually sealed in polyethylene bags and stored at −34°C until analyzed for chlorophyll and yellow pigments. One disc from each prosition was used for determination of chlorophyll and the other for yellow pigments.

On the same day the fruit was harvested, that portion of each sample not retained for pigment measurement was squeezed through Mira-

Table 1 – Variable ranges[a] for peaches of three cultivars

Variables	Range from immature to ripe by cultivar					
	Keystone		Loring		Nectar	
DLE, volts	0.88	0.05	0.90	0.07	0.90	0.10
L	70.97	39.17	67.73	41.10	67.07	39.33
a	−7.60	32.97	−6.37	30.07	−10.03	33.80
b	32.53	13.43	32.17	12.77	27.70	11.30
θ, degrees	104.09	25.56	101.43	25.68	110.09	20.56
Firmness, Newtons	73.39	0.89	58.94	3.34	72.28	2.22
Chlorophyll, μg/g	15.25	1.71	10.80	2.20	16.25	0.50
Soluble solids, %	9.20	11.92	9.63	15.03	8.77	12.97

[a] Values except firmness are means of measurements at three positions on each fruit; firmness values are means of two positions.

Table 2—Simple correlation coefficients[a] of variables for peaches of three cultivars

	Average maturity rank	Firmness	Chlorophyll	Soluble solids	Yellow pigments	L	a	b	θ	Ground color a
DLE	−0.94	0.89	0.74	0.59	0.16	0.84	−0.89	0.80	0.89	−0.87
Average maturity rank		−0.90	−0.84	0.57	−0.22	−0.82	0.91	−0.77	−0.90	0.90
Firmness			0.74	−0.51	0.35	0.75	−0.82	0.74	0.81	−0.78
Chlorophyll				−0.44	0.38	0.64	−0.72	0.63	0.73	−0.65
Soluble solids					−0.27	−0.59	0.56	−0.57	−0.59	0.57
Yellow pigments						0.33	−0.16	0.52	0.20	−0.05
L							−0.87	0.96	0.92	−0.83
a								−0.82	−0.98	0.89
b									0.89	−0.74
θ										−0.87

[a] $p < 0.01$ for $r > 0.29$; $P < 0.05$ for $r > 0.23$.

Table 3—Analysis of variance F values for cultivar and position effects on variables evaluated for peaches of three cultivars

Variable	Cultivar	Position	Cultivar × position
DLE	3.86*	2.65	0.38
L	1.42	151.48**	0.10
a	0.33	19.74**	1.36
b	12.71**	144.32**	0.57
θ	0.42	48.52**	0.68
Firmness	1.23	ND	ND
Chlorophyll	3.28**	1.40	2.15
Soluble solids	14.24**	9.69**	1.50

* Significant at the 95% level.
** Significant at the 99% level.
ND = Not determined.

cloth in a garlic press to obtain a juice sample. The percent soluble solids in the sample was read directly with a Bausch & Lomb refractometer. All core samples were removed from the −34°C storage and analyzed for chlorophyll and yellow pigments within 60 days of their harvest date. Chlorophyll was determined using a modification of the method described by Harborne (1973). The tissue discs were macerated in 80% acetone with $CaCO_3$ for stabilization, filtered, made to volume and the A_{652} read in a grating spectophotometer. For determination of yellow pigments the tissue discs were macerated in 60:40 hexane/acetone and the calculated concentration was based on the A_{472} value.

Statistical analysis

Analysis of variance was performed on the data set using the GLM procedure of SAS (1982) to evaluate cultivar and position effects and interactions. Cultivar and position means were separated using Least Significant Difference (LSD) procedures. All possible correlations between variables were computed. In order to estimate relative maturity of the 35 fruits of each cultivar, they were ranked from most immature to ripest on the basis of values obtained for each variable evaluated, except yellow pigments. Yellow pigment values were not used for estimating maturity because their correlations with the other variables were low. For each cultivar an average maturity rank for each fruit was calculated by averaging the ranks for all variables. This calculated average maturity rank was an indicator of the relative maturity of fruits of each cultivar. The relationship between DLE and average maturity rank for each cultivar was evaluated by regression analysis.

RESULTS & DISCUSSIONS

THE RANGE of values obtained for the variables (Table 1) shows the magnitude and direction of changes that occur for each variable during the ripening of peaches. Hunter L, a and b values showed there were decreases in lightness and amounts of green and yellow and an increase in redness. The decrease in θ also indicated a change in visual color appearance from greenish to reddish. DLE, firmness, and chlorophyll decreased during ripening while values for soluble solids increased. Chlorophyll content and firmness as determined by pressure tests have been reported to decrease with ripening of peaches (Reed, 1957). DLE correlated reasonably well with all variables known to be related to peach maturity except yellow pigments (Table 2).

There were significant differences among the peach cultivars evaluated in DLE, Hunter b, chlorophyll and soluble solids values (Table 3). DLE and soluble solids were significantly higher for Loring peaches than for Keystone and Nectar (Table 4). Keystone had significantly higher chlorophyll and lower soluble solids than Loring and Nectar. Keystone and Loring had significantly higher Hunter b values than the white-fleshed Nectar indicating more yellow coloration on their external surface.

DLE and chlorophll were not significantly different among positions on the fruit (Table 3). All color measurements varied significantly by fruit position as expected, but percent soluble solids was the only chemical property that varied significantly among positions. Soluble solids measurements taken on the ground color area were significantly lower than on the blossom end or the blush area (Table 4). The lower values of a and higher values of θ for the ground color area indicated a more green color. Also the higher L and b values for that area of fruit surface indicated a lighter and more yellow appearance than for the other fruit positions. Conversely, the higher a value for the blush area and blossom end indicated these positions had a more red appearance.

The regressions of DLE on average maturity rank for peaches of the three cultivars are given in Fig. 1–3. The observed values of DLE versus average maturity rank for the fruits were plotted around the regression lines. The prediction equations

Table 4—Cultivar and position means for physical and chemical properties of peaches

	Cultivar means			Position means		
Variables	Keystone	Loring	Nectar	Blossom end	Blush area	Ground color area
DLE, volts	0.46b	0.55a	0.47b	0.48a	0.46a	0.54a
L	55.16a	53.65a	53.01a	53.36b	42.89c	65.57a
a	14.05a	15.45a	14.33a	17.31a	18.49a	8.02b
b	23.42a	22.28a	19.37b	21.67b	14.65c	28.76a
θ, degrees	57.99a	54.71a	55.46a	53.59b	39.06c	75.52a
Firmness, Newtons	37.98a	37.27a	29.98a	ND	ND	ND
Chlorophyll, μg/g	6.25a	5.27b	5.22b	5.33a	6.02a	5.39a
Soluble solids, %	10.56c	11.57a	11.05b	11.39a	11.20a	10.59b

[a-c] Cultivar means are based on measurements made at 3 positions on each of 35 fruits and position means are based on measurements made at one position on 105 fruits. Means with the same letter in the same row for cultivars and positions are not significantly different according to LSD, 5% level.
ND = Not determined.

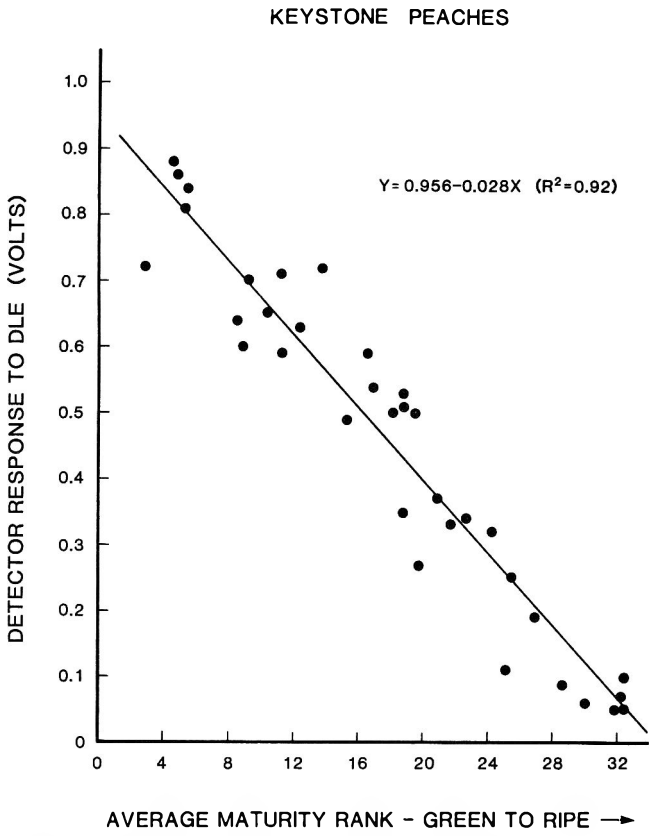

Fig. 1—Regression of DLE on average maturity rank for Keystone peaches.

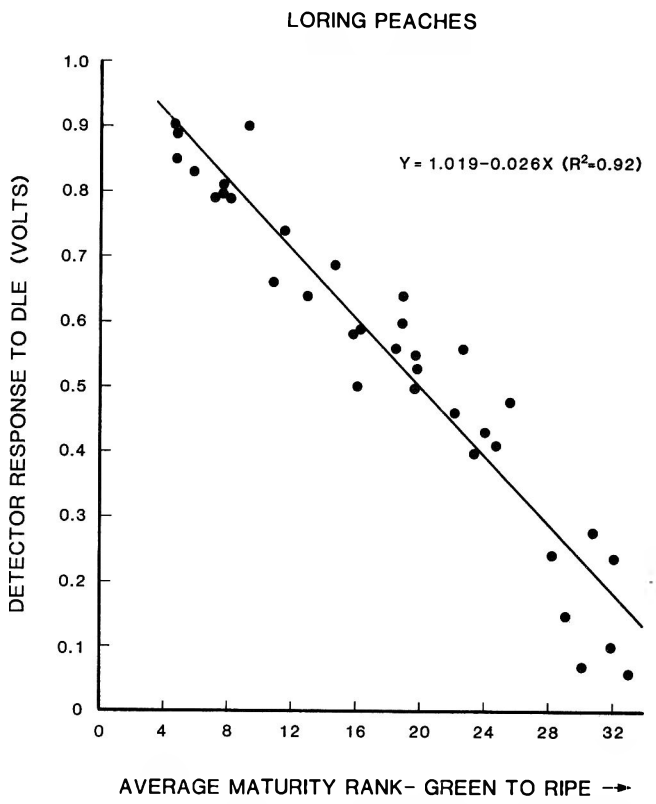

Fig. 2—Regression of DLE on average maturity rank for Nectar peaches.

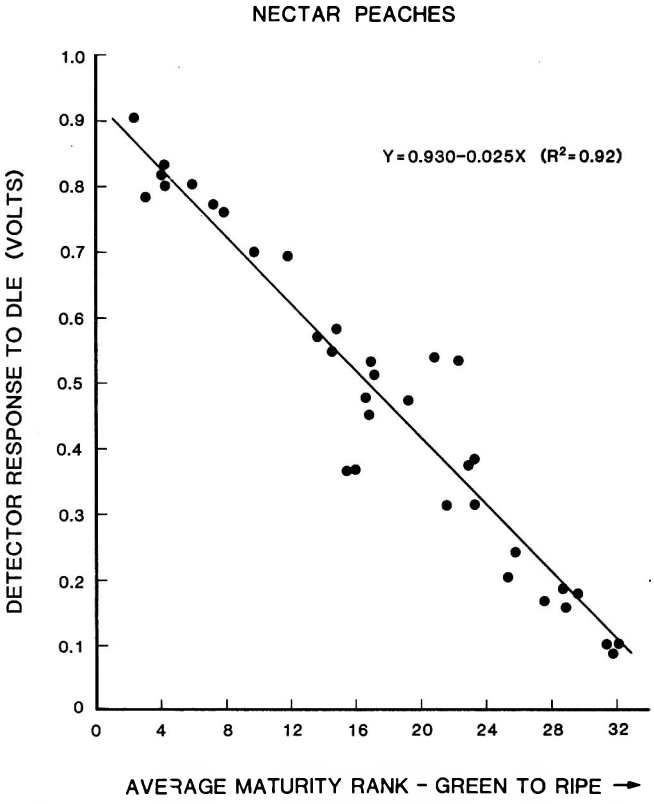

Fig. 3—Regression of DLE on average maturity rank for Loring peaches.

for all three cultivars had R^2 values of 0.92, indicating a high correlation between DLE and relative maturity. DLE decreased linearly with average maturity rank for the three cultivars. The R^2 values for the regressions of ground color a value, (commonly used as an indicator of maturity) on average maturity rank were 0.87 for Keystone and Loring and 0.73 for Nectar. Thus average maturity rank was an effective indicator of peach maturity.

The relationship between DLE and average maturity rank for peaches of the three cultivars can be compared by analyzing the terms of the prediction equations given in Fig. 1–3. The intercept in the prediction equation for Loring peaches was greater than the intercepts in the equations for the other cultivars which indicated that Loring had higher initial DLE values than the other two. The slopes in the equations are about the same indicating DLE decreased with average maturity rank at about the same rate for all three cultivars. Since Loring had higher DLE values initially, those values were higher than the other fruit over the full maturity range. This was interesting because Loring did not have the highest level of chlorophyll, thus indicating other factors in addition to chlorophyll might contribute to DLE production.

Our study is a continuation of work at this location to develop a rapid and nondestructive technique for measuring maturity of fruits and vegetables applying the principle of DLE. Results of this study were similar to results obtained in previous studies with tomatoes (Forbus et al., 1985), papaya (Forbus et al., 1987) and cantaloupe (Forbus and Senter, 1989) in that DLE highly correlated with chemical and physical properties shown to be related to maturity.

Results obtained in our study with peaches differed from results obtained with other products in that neither DLE nor chlorophyll varied significantly among positions on the fruit. In previous work with papaya, DLE and chlorophyll measurements varied between positions on the same fruit by as much as 75% and 60%, respectively. The variation in these properties by position on the fruit meant that, in using DLE to predict papaya maturity, the position where the measurement was made on the fruit was important. In such cases, in applying DLE in an automated sorting system the fruit would have to

be oriented to a known position. Because DLE did not vary significantly by position in these three peach cultivars DLE measurements at any position could be used to predict peach maturity. In an automated sorting system elimination of the need to orient fruit would simplify design and reduce cost of equipment required.

High correlations between DLE and physical and chemical properties known be related to peach maturity indicate DLE could provide a rapid, nondestructive method for measuring peach maturity at all stages of marketing. Additional work is needed to optimize conditions for applying DLE to measure peach maturity under commercial operating conditions.

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goes during ripening was noticeable as compared to the controls where no decrease was evident. A decrease in palmitic acid caused a change in the palmitic/palmitoleic ratio. This change was parallel in both cases (Table 1 and 2). A marked decrease in linoleic content of pulp oil was observed in unirradiated fruit on the 6th day of storage compare with irradiated fruit, where linoleic content remained unaffected up to 12 days of storage. A gradual increase in linolenic acid in the pulp oil up to 1.5 fold for unirradiated fruits was observed compared to 2.0-fold increase for irradiated fruits (Table 1). A parallel decrease in ratio of oleic/linolenic acid was observed in both unirradiated and irradiated fruit during ripening; however, in the case of irradiated samples this ratio reduced to half that of unirradiated fruits in the advanced stage of ripening (Table 2). Thus, irradiated mangoes stored 15 days gave values similar to unirradiated fruit stored 12 days indicating a 3 day delay in ripening (Table 2). Also in case of irradiated mangoes even after 12 days storage the sensory evaluation showed the fruits were in half-ripe state as compared to controls in which the same state was attained on 6 days storage (Table 2).

Our results indicate gamma-irradiation (0.25 kGy) caused delay in ripening by at least three days without causing any effective changes in volatile GC profiles.

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Variability in the Quantities of Condensed Tannins and Other Major Phenols in Peach Fruit During Maturation

SAMUEL D. SENTER and ANN CALLAHAN

ABSTRACT

Quantities of condensed tannins and the major monomeric phenols were maximum between the first and second swell of fruit growth in six melting flesh peach cultivars. Quantities of tannins were higher and vanillin-proanthocyanidin ratios were lower in low quality, astringent fruit than in white or yellow flesh, commercial quality fruit. Major monomeric phenols in all cultivars were chlorogenic acid, neochlorogenic acid, isochlorogenic acid, catechin, and epicatechin. Quantities of these compounds varied by cultivar and also were greater in the low quality, astringent fruit between the first and second swell of growth.

INTRODUCTION

THE GOAL OF PEACH BREEDING programs has long been to develop cultivars (cvs.) that exhibit both cold hardiness and fruit quality. Many generations of hybridization and selection are required to combine favorable alleles for both characteristics. Such difficulties are currently being overcome by advances in plant regeneration, molecular biology, and gene transfer. Manipulation of specific traits through genetic engineering allows one to selectively manipulate genes without the necessity of the sexual cycle and recombination of genes that results from that process. In peach breeding, the genetic manipulation of specific aspects of fruit quality is considered more feasible than manipulation of cold hardiness where physiological and genetic relationship are not known.

Peach quality is a general term encompassing size, color, texture, flavor, aroma and other factors. Many of the biochemical properties constituting these characteristics appear to be single gene loci controlled and have moderately high heritability (Callahan et al., 1989). Biochemical studies of peach fruit indicate levels of acids, sugars, soluble solids and polyphenols are the primary quality parameters that differ among cvs. (Sistrunk, 1985). When these parameters of quality are compared between commercially acceptable and unacceptable genotypes, only condensed tannin levels appear to correlate with these two fruit classes (Robertson et al., 1988).

Condensed tannins are important to fruit astringency and flavor. (Sistrunk, 1985). Loss of astringency and thus improvement in palatability and quality in developing fruit are related to changes in tannin composition and quantity. Reeve (1959) observed marked changes in tannin content of peaches during development and ripening and observed with histological studies that these compounds were localized in enlarged mesocarp cells. Their appearance was coarse and granular in green fruit and became less intense and more divided with ripening. In contrast to that study, Goldstein and Swain (1963) did not observe reductions in tannins during ripening and noted the distribution of tannins throughout the tissue. Climactic conditions have a marked effect on quantities of tannins in peaches (Guadagni and Nimmo, 1953) with warm and clear weather resulting in lowest tannin content. Kader and Chordas (1984) found large differences in browning potential of peaches within and among cvs. and related these to total phenol content and polyphenoloxidase (PPO) activity as did Grice et al. (1952).

Condensed tannins are indigenous to woody plants and are formed from an initiating molecule of catechin (flavan-3-ol) and extension units of enzymatically condensed flavan-3, 4-diols commonly referred to as proanthocyanidins (Stafford et al., 1986). Decreased astringency of fruit is thought to relate to increased size of polymers forming the condensed tannins (Goldstein and Swain, 1963; Porter and Woodruffe, 1984) and through size exclusion to prohibit interaction of the molecules with the proline-rich salivary protein (Mehansho et al., 1987). Tannins with estimated molecular weights of 500–3000 are implicated in astringency sensation and involve only those polymers containing 2-6 monomers (Ribereau-Gayon, 1972, Haslam, 1975).

The objective of our study was to provide basic data on the formation of condensed tannins and related phenolic compounds in peach fruit during maturation. We also wanted to provide information for use in breeding programs to develop cold hardy cvs. that produce commercial quality fruit, by making quantitative and qualitative comparisons of these compounds between low quality, astringent fruit from cold hardy cvs. and yellow and white flesh market quality fruit.

MATERIALS & METHODS

Fruit samples

Fruit from low quality, astringent (Bailey and Boone County), yellow-flesh, intermediate quality (Reliance), yellow-flesh, commercial quality (Loring) and white-flesh, commercial quality (Belle of Georgia) peach cvs. were obtained from the U.S. Dept. of Agriculture's Appalachian Fruit Research Station during the 1987 growing season. Selections from each cv. were made from several trees that had received normal horticultural care. The number of fruit obtained at each sampling varied but was sufficient to adequately represent fruit development at the particular stage of maturation. Time between samplings varied by cv. within 160 days of development after flowering and was based on fruit size and stage of development rather than predetermined time intervals. The fruit were frozen and lyophilized immediately after collection then stored in polyethylene bags under low temperature (0.°C), desiccated conditions. Prior to analysis, the exo-and mesocarps were removed from the endocarps and then compositely by sample date and cv. The tissue was ground with a mortar and pestle and stored in glass jars under desiccated conditions.

Condensed tannin analysis

Phenolic compounds were extracted from the lyophilized peach tissue in two stages. Five hundred mg tissue was first ground in about 40 mL methanol: water (70:30, V/V) with a tissue homogenizer and shaken 1 hr with a wrist-action shaker. The mixture was transferred to tubes for removal of particulates by centrifugation after which the supernatant was decanted and made to 50 mL volume. The residue was quantitatively transferred to screw-capped tubes with 10 mL of the aqueous methanol and refluxed 30 min to solubilize those phenolic compounds not extractable at ambient temperature. After cooling to ambient, the supernatant was again made to volume with extracting solvent.

Condensed tannins were estimated by determination of both proan-

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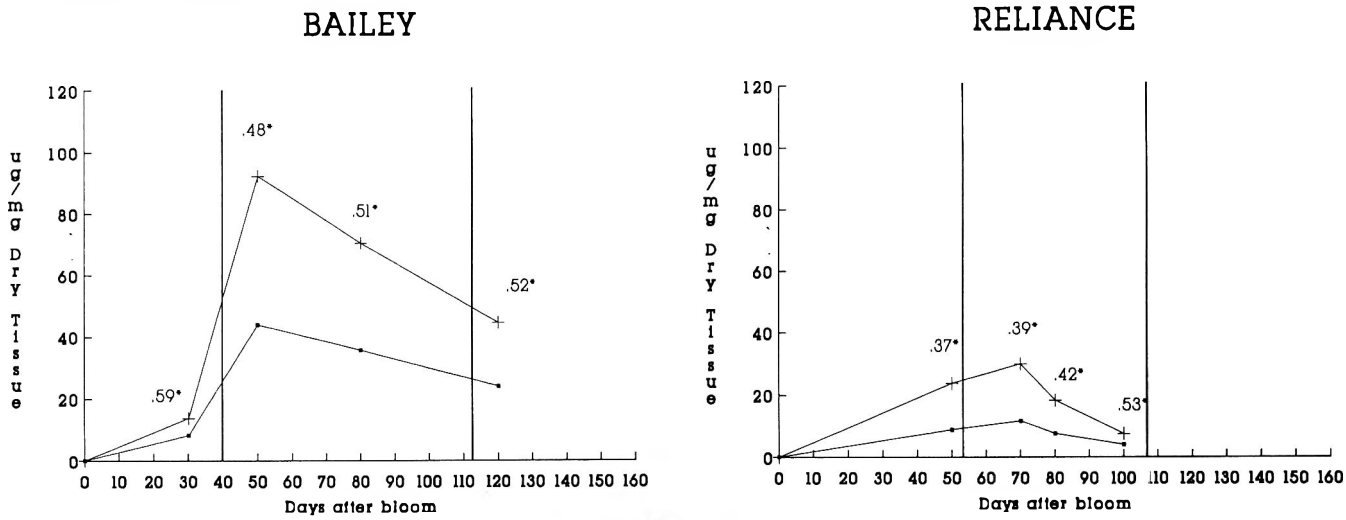

Fig. 1-2—Representative proanthocyanidin (--+--) and vanillin (--■--) values and V/PA ratios (*) of astringent (Bailey) and nonastringent (Reliance) peaches during maturation. Values represent the total extractable tannins. Vertical lines indicate time of 1st and 2nd swell in fruit growth.

Table 1—Proanthocyanidin (PA) and vanillin (V) values of astringent, low quality (Boone County), yellow (Loring) and white (Belle of Georgia) flesh, commercial quality peaches during maturation

Cultivar	Time[b]	Extract[c]	µg/mg[a] PA	V	V/PA
Boone County	56	E	26.00	27.32	1.05
		R	23.04	0.47	0.02
	84	E	65.00	45.52	0.72
		R	39.78	1.50	0.04
	111	E	70.00	40.00	0.57
		R	30.96	1.04	0.03
	125	E	41.00	31.65	0.77
		R	28.08	0.67	0.02
	140	E	39.00	21.80	0.56
		R	16.92	0.41	0.02
Loring	62	E	7.00	9.97	1.42
		R	10.44	0.57	0.05
	89	E	12.00	11.46	0.96
		R	10.98	0.43	0.04
	102	E	9.00	8.72	0.97
		R	5.76	0.20	0.03
	120	E	17.00	9.31	0.55
		R	5.22	0.25	0.05
Belle of GA	57	E	14.00	9.96	0.71
		R	13.50	0.36	0.03
	83	E	25.00	22.18	0.89
		R	14.94	0.44	0.03
	110	E	16.00	15.13	0.95
		R	14.94	0.43	0.03
	131	E	8.00	4.41	0.55
		R	3.06	0.19	0.06

[a] Dry weight basis
[b] Days after bloom
[c] E = Extraction at ambient temp with 70:30 methanol-water and sonication; R = Reextraction of tissue (E) at 100°C with 70:30 methanol-water.

thocyanidin content and vanillin values of the extracts. Proanthocyanidin content of one mL of a 1:10 dilution of the extracts was determined by measurement of optical density at 535 mm after reaction with butanol-HCl-$FeSo_4$ reagent for 1 hr at 97°C (Swain and Hillis, 1959; Mole and Waterman, 1987). Quantities of anthocyanidins per mL of solution were determined with a spectrophotometer using a "K" factor established with cyanidin-HCL (Extrasynthese, 69730 Genay-France) with subsequent conversion of quantities to µg/mg dry tissue with appropriate conversion factors. Vanillin values were determined on 1 mL of a 1:10 dilution of the extracts after reaction with vanillin-H_2SO_4 reagent (Swain and Hillis, 1959). Optical densities of solutions were measured at 500 nm and concentrations of flavanols/mL solution were determined with a spectrophotometer using a "K" factor determined with a catechin standard. Appropriate conversion factors were used to convert these quantities to µg/mg dry tissue.

HPLC analysis

Monomeric phenols were extracted from 500 mg of lyophilized tissue with 5 mL methanol and sonication as previously reported (Senter et al., 1989). After 5 min extraction, the solutions were prepared for analysis by centrifugation, filtration and bringing to 5 mL volume with extracting solvent. One hundred µg of methyl-4-hydroxybenzoate was added as an internal standard. Separations of 6 µL injections of the extracts were made with an 8 cm, C_{18} reverse phase, Pecosphere column (Perkin Elmer, three micron particle size). A linear gradient of methanol-water, each containing 0.1% H_3PO_4, was developed in 20 min with a Perkin Elmer model 410 HPLC pumping system. The gradient was from 5–95% methanol:water at a flow rate of 1 mL/min. The eluted compounds were detected with a Perkin Elmer Array Detecting System which also provided UV spectra of the compounds. These were identified by comparison of spectra and retention times with authenticated compounds and quantitated with a Perkin Elmer computing integrator by comparison of peak areas with the internal standard.

RESULTS & DISUSSION

THESE DATA indicate differences exist in the quantities of condensed tannins and simple phenols in peaches, both as a function of maturation and by cv. Deposition of these compounds began in the early stages of development in all cvs. evaluated and increased to a maximum between the first and second swell in fruit growth (Fig. 1 and 2). The time of maximum deposit varied by cv. within this time frame, but in all cvs., declines began in the interim between the first and second swell of fruit growth which corresponded roughly to the threshold mature stage of fruit development. Differences in quantities of condensed tannins by cvs. were readily apparent by comparison of PA and V values in Fig. 1–2 and Table 1. Fruit from the Boone County and Bailey cvs. contained 2–3x more tannins than fruit from the intermediate and commercial quality cvs. which probably contributes to the low quality, astringent charcteristics of these cvs. The lower PA and V values of the intermediate and commercial quality fruit were comparable among cvs. with total contents of about 40 µg/mg tissue being measured by the PA analyses and about 30 µg/mg tissue by the vanillin analyses. V to PA ratios are indicated at the sample dates in Fig. 1–2 and in Table 1. Changes in these values were noted by cv. with maturation and could be related to lesser degrees of polymerization (Goldstein and Swain, 1963) although this is highly unlikely. This becomes apparent with critical examination of Table 1 which displays the PA and V values and their ratios in relation to extraction procedures.

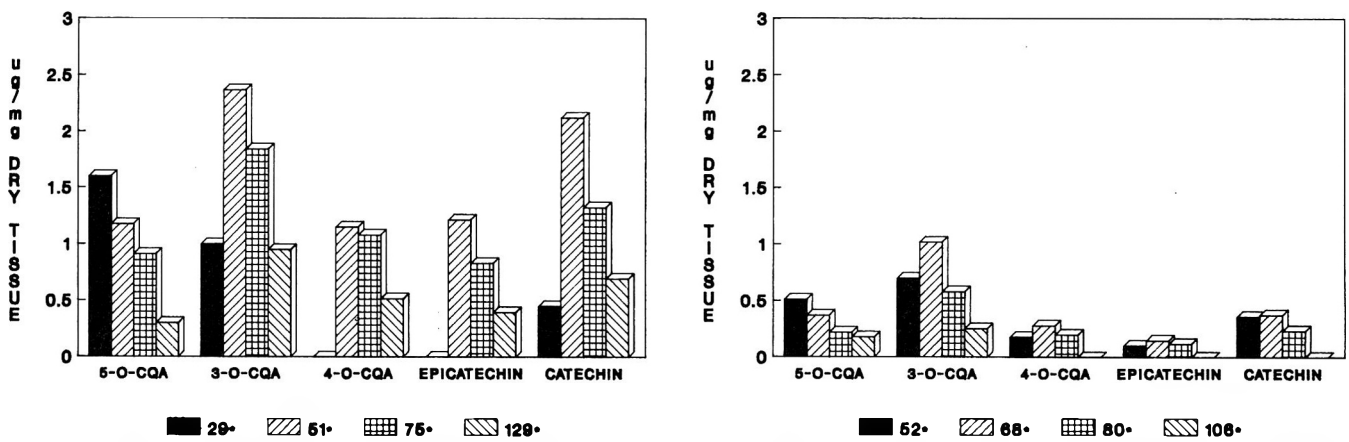

*Fig. 3-4—Representative changes in the quantities of the major monomeric phenols in astringent (Bailey) and nonastringent (Reliance) peaches during maturation. [a]5-0-CQA = neochlorogenic acid, 3-0-CQA = chlorogenic acid, 4-0-CQA = isochlorogenic acid. * Number of days after bloom when sampled.*

Table 2—Variability in quantities of major polyphenols in low quality astringent (Boone County), yellow (Loring) and white (Bell of GA) flesh, market quality peaches during maturation

		µg/mg[a]				
Cultivar	Time[b]	3-0-CQA[c]	4-0-CQA[c]	5-0-CQA[c]	Catechin	Epicatechin
Boone County	56	2.16	0.00	2.66	1.24	0.54
	84	2.30	0.59	1.22	1.68	1.14
	111	1.61	1.10	0.84	1.18	0.91
	140	0.70	0.47	0.38	0.23	0.40
Loring	62	0.47	0.65	0.00	0.41	0.12
	89	0.43	0.31	0.00	0.46	0.24
	102	0.26	0.17	0.00	0.30	0.14
Belle of GA	57	0.45	0.63	0.00	0.37	0.00
	83	0.69	0.29	0.65	0.69	0.33
	110	0.52	0.46	0.27	0.40	0.24
	131	0.21	0.19	0.00	0.13	0.00

[a] Dry weight basis.
[b] Days after flowering.
[c] Isomers of chlorogenic acid.

Fairly close approximations were observed in the quantitation of tannins by the two methods in the ambient 70:30 methanol:water extracts (extract E) and the ratios of their values were in the range of those reported by Goldstein and Swain (1963). The ratios tend to decline with time and indicate increased polymerization. However, when the tissue was re-extracted with boiling aqueous methanol, the vanillin values for these extract (extract R) were much lower and not in compliance with determined proanthocyanidin values. The ratios of these values are therefore not reliable in this study for predicting degree of polymerization. We could not explain the discrepancy between the PA and V values in extracts R of Table 1. However, the formation of anthocyanidins from the colorless extracts with butanol-HCl indicated that anthocyanidin precursors were present in the peach tissue, probably as complexes with carbohydrates and/or proteins, and were liberated only with extraction at elevated temperatures. The low response of these extracts to the vanillin assay indicated the probable presence of monomeric compounds (flavan-3,4-diols) since this test was reported relatively insensitive to monomeric flavanols in methanol (Butler et al., 1982).

The presence of the three chlorogenic acid isomers previously reported by Swain (1962) as peach constituents was confirmed. Their identity, tentatively established by UV spectra, was confirmed by coinjection with methanolic extracts of *Nicotiana* goodspeedii which was shown by Snook et al. (1986) to contain appreciable quantities of neochlorogenic, chlorogenic and isochlorogenic acids. These isomers, in addition to catechin and epicatechin, constituted the principal monomeric phenols in peaches. The anthocyanidins, cyanidin and delphinidin, were observed as traces in some extracts and their absence was attributable to the immature development stage of the sampled fruit. The same pattern of concentration by cv. and time was apparent in these analyses as was observed in the analysis of condensed tannins (Fig. 3–4, Table 2). Higher concentrations were found in Boone County and Bailey peaches with much lower quantities in commercial quality fruit. Variability in quantity was observed by cv. within the low quality, astringent cvs. and also in the other cvs. sampled. Boone county peaches contained about twice as much neochlorogenic acid in the early stages of development as fruit from the Bailey cv., whereas, this compound was absent from Loring cv. at all stages of sampling. In general, little quantitative difference was observed in the other compounds in the commercial quality fruit. Concentrations in relation to time were consistent with deposition of condensed tannins in all cvs. i.e. maximum concentrations occurrred between first and second swell in growth and declined thereafter.

The quantitative variance with time in formation of phenolic compounds in peaches, as shown in our study, provides opportunity to optimize time in relation to genetic modification through bioengineering. Research can be conducted at the time of maximum enzyme activity in low quality, astringent peach cvs. for development of cvs. with both cold hardiness and market quality. The need for modification and improved interpretation of results from standard analytical procedures are needed for studying condensed tannins. The applicability of vanillin-proanthocyanidin ratios for estimating degree of polymerization of condensed tannins was shown to be limited.

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Significance of Copper in Alcohol Production with Fermentation of Raisin Extracts by the Cell Recycle Process

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ABSTRACT

The ethanol fermentation rate, cell growth and yield were reduced from one batch to the next in presence of 50 ppm copper as compared to 5 ppm. The decreases of these parameters are attributed to the increased uptake of copper causing lessening of the glucose uptake by yeast cells. These changes were studied through the uptake of ^{14}C-glucose in fermentations of synthetic media containing ^{14}C-glucose, in presence of various copper concentrations.

INTRODUCTION

THE CELL RECYCLE PROCESS (Fig. 1) is widely employed in production of potable alcohol, because recycling the same yeast culture from batch to batch gives satisfactory ethanol yields and productivity. In this process, the fermentability of raw materials is related to the number of repeated batch fermentations undergone by the yeast culture. That affects mainly the ethanol yield, or productivity, and depends on the composition of the fermentation broth. Raisins as a raw material are widely used in Greece for ethanol production. The cultivars mostly used are *Trechumena, Piotiki dialogi, Chondrada, and Psila*. Only *Trechumena* is consumed as food; all these cultivars are used for potable alcohol production using the repeated batch system. *Trechumena* may result in the best fermentation whereas *Psila* the worst. In a previous study (Akrida-Demertzi et al., 1988) *Trechumena* was reported to exhibit low copper content and *Psila* higher. Therefore, we believed that the reduction of ethanol productivity observed in industry with the repeated batch fermentations of raisin extract from *Psila* was due to its relatively higher copper.

Although many researchers (Cook, 1958; Grafl and Schawantes, 1958; Veliky and Jozei, 1964; Khrycheva, 1970; Failla et al., 1976; Heldwein et al., 1977; Joho et al., 1983, 1985; Berthe-Corti et al., 1984) studied the effect of some trace elements on yeast cells and fermentation, such studies concerning the trace element copper have not been reported. Likewise, the uptake of some divalent cations by yeast cells has been studied in the past (Noris and Kely, 1977; Roomans et al., 1979; Eilam, 1982). However, the uptake of copper has not been reported from batch-to-batch in repeated batch fermentations in relation to cell growth, ethanol production rate, and yield. Especially, this was not done for synthetic media containing fermentable sugar or for raisin extracts and related raw materials for potable alcohol production. Therefore we set the objective to examine the effect of copper on ethanol productivity, yield, and cell growth obtained from batch-to-batch fermentations of raisin extract.

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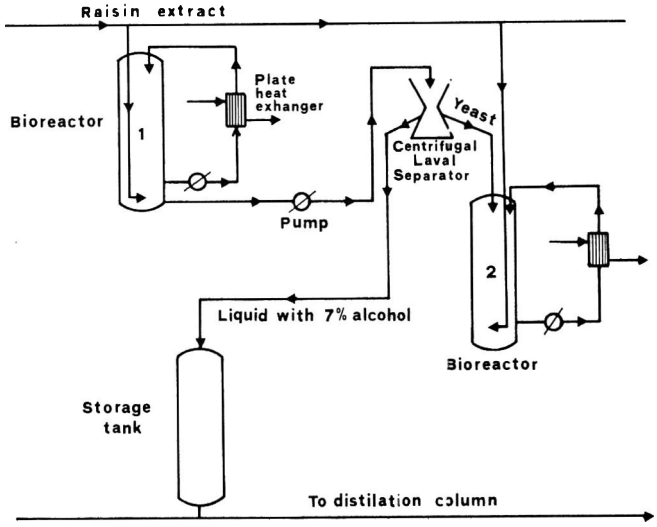

Fig. 1—Cell recycle process diagram for potable alcohol production with raisin extracts.

MATERIALS & METHODS

Preparation of raisin extracts

Greek raisins, cultivar *Trechumena*, were used. A 5-L Erlemeyer flask, containing the appropriate amounts of pulpified raisin and tap water, was placed in a 72°C water bath for 6 hr. The weight proportion raisin/water was set according to the invert-sugar in the raisin extracts obtained and the pH was adjusted to 3.2 with sulfuric acid.

Repeated batch fermentations of raisin extracts

A fermentor of 1L capacity was used and fermentations were performed without stirring. The fermentor contained 500 mL raisin extract, of density 7°Be and a copper sulfate solution was added yielding copper concentration of 50 ppm. Ten grams pressed Baker's yeast, *Saccharomyces cerevisiae*, were added to the flask and the mixture was incubated at 30°C. At the end of the first batch the yeast culture was separated by centrifuge and an amount of it was used for the second batch, so that the cell concentration as well as the copper content would be similar to those of the first batch. This was repeated for batches that followed.

During the fermentations, measurements were made of °Be density at various time intervals for kinetic study and optical density for the determination of cell growth. After fermentation the liquid was analyzed for ethanol, biomass and residual sugar and samples of the separated yeast were tested for amount of copper uptake by yeast cells. Also, a parallel fermentation of Trechumena extract containing 5 ppm copper was performed.

All values of fermentation time were the mean of three replicates and the standard deviation was $< \pm 0.5$.

Batch fermentations of synthetic media containing sucrose

The cultures used in sucrose batch fermentations were prepared as follows: Using Baker's yeast a colony was isolated and allowed to grow on complete medium containing yeast extract agar (Gutz et al.,

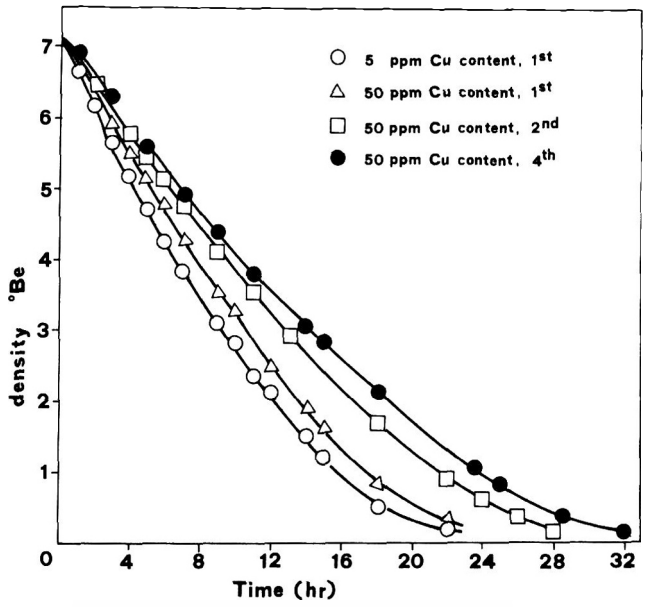

Fig. 2—Fermentation kinetics of raisin extract containing 50 ppm Cu in repeated batch fermentations, as compared with that of 5 ppm Cu.

Table 1—Copper uptake by Saccharomyces cerevisiae *cells in the alcoholic fermentation of raisin extracts by cell recycle, as related to ethanol yield, productivity and cell growth*

Batch no.	Copper (ppm) Conc	Uptake	Ethanol fermentation Time (hr)	Productivity[a]	Yield[b]	Growth g·L⁻¹
1	5	—	22	56.3	0.47	30.0
		203[c]				
1	50	602	22	56.3	0.47	22.0
2	50	953	28	45.8	0.45	17.0
3	50	1276	29	44.4	0.44	16.0
4	50	1413	32	37.5	0.43	11.0

[a] g/L·24 hr
[b] g ethanol/g sugar utilized
[c] In the case of raisin extract fermentation baker's yeast was used.

1974). This was inoculated into a minimal liquid medium, in order to obtain the appropriate biomass for one batch fermentation.

For batch fermentations of sucrose, solutions of 7°Be density were employed with a cell concentration of *Saccharomyces cerevisiae* equal to 20g/L. Also, several batches containing different concentrations of copper (obtained by copper sulfate addition) were used at 30° C in these experiments.

During the fermentations the cell growth at various time intervals was determined. After the fermentation the ethanol, biomass, residual sugar and uptake of copper by the yeast cells were analysed.

Glucose uptake by cells

Certain experiments were organized using ^{14}C-glucose to find some relation of glucose uptake rate to concentration of copper. A culture of *Saccharomyces cerevisiae*, was prepared by a mono-cell culture as described with various concentrations of copper. Aerobic fermentations were carried out using solutions containing various glucose concentrations to which 0.5 μCi ^{14}C-glucose/50 mL and a given amount of yeast cells (the same in all experiments) were added. This was done to determine the concentration of glucose at which the highest uptake of ^{14}C-glucose was obtained (found to be 1000 nM). At that concentration aerobic fermentations were performed in the presence of ^{14}C-glucose, using the following concentrations of copper (by adding copper sulfate): 0, 0.2, 1, 3, 5, 7, 10, 15, and 30 ppm. In each fermentation corresponding to a copper level, as well as those to study optimum glucose, a sample of yeast dry cells was collected after filtration, using Sartorius filter 0.45μ, washing and drying at room temperature. The dry biomass was added, in 5 mL toluene solution containing 4 g L^{-1} PPO (2,5 diphenyloxazole) and 0.1 g.L^{-1} POPOP [1,4 Bis [2-(5-phenyl)-oxazolyl] benzol] (Dramas et al., 1982) and the ^{14}C-glucose measured employing liquid scintillation counter, Intertechnique SL 3000. The glucose uptake rate was calculated as nM glucose per min per mg dry weight *Saccharomyces cerevisiae* cells.

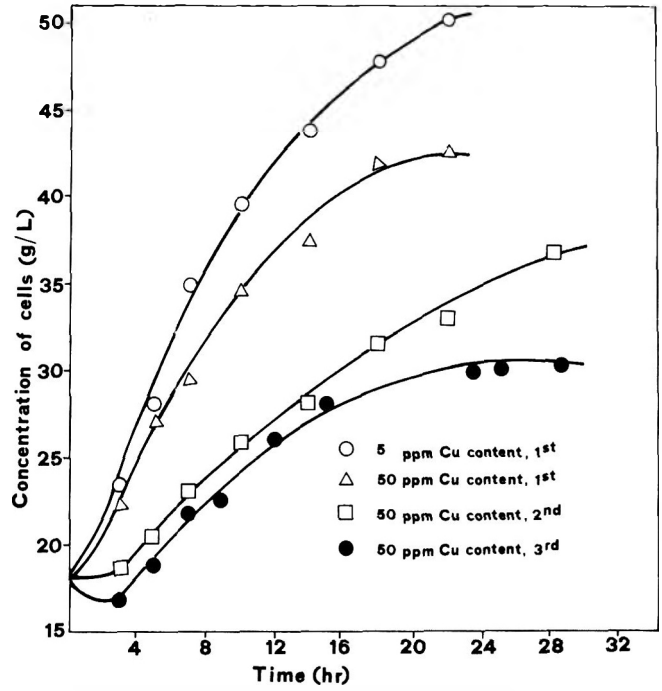

Fig. 3—Biomass wet weight vs time during repeated batch fermentations of raisin extract containing 50 ppm Cu, as compared with that of 5 ppm Cu.

Uptake of copper by cells

Copper uptake by yeast cells was determined by atomic absorption spectrometry, using a Perkin Elmer, model 560 spectrophotometer, equipped with an acetylene-air flame and copper hollow cathode lamp. The analysis was made on the ash obtained after combustion of the yeast cells at 550°C in a muffle furnace. The yeast cells were separated by centrifugation of fermenting liquid.

All values of copper uptake were the mean of three replicates and standard deviation was < ± 20.

Ethanol, biomass and sugar determinations

The ethanol concentration was determined using a Varian 3700 gas liquid chromatograph, Porapac S as column material and Nz carrier gas (40 mL/min). The ethanol productivity was reported as grams ehtanol/L produced after 24 hr. The ethanol yield factor is the ratio of g ethanol/g sugar utilized.

The biomass was determined by measuring the optical density at 700 nm. Standard curves of optical density vs wet weight (g/L) cell were prepared for estimation of the biomass concentration in the sample.

In order to calculate the ethanol yield factor on the basis of sugar used by the cells, the residual sugar of the fermentation product was determined. Also, the residual invert-sugar of the liquid obtained after fermentation of raisin extracts was analyzed, using the well known Lane Eynon analytical procedure (Egan et al., 1981). Residual sucrose was also estimated by the same method, after its hydrolysis with hydrochloric acid 37%, heated in a water bath for 30 min.

All values were the mean of three replicates. The standard deviation for yield was < ± 0.003 and for biomass < ± 0.2.

RESULTS

THE IMPORTANT DIFFERENCES observed in the fermentability of *Trechumena* and *Psila* extracts, was attributed to greater concentrations of copper of *Psila* (Akrida-Demertzi et al., 1988). In order to verify that increased concentrations of copper, higher than those contained in *Trechumena*, reduces ethanol production from batch-to-batch, fermentations of *Tre-*

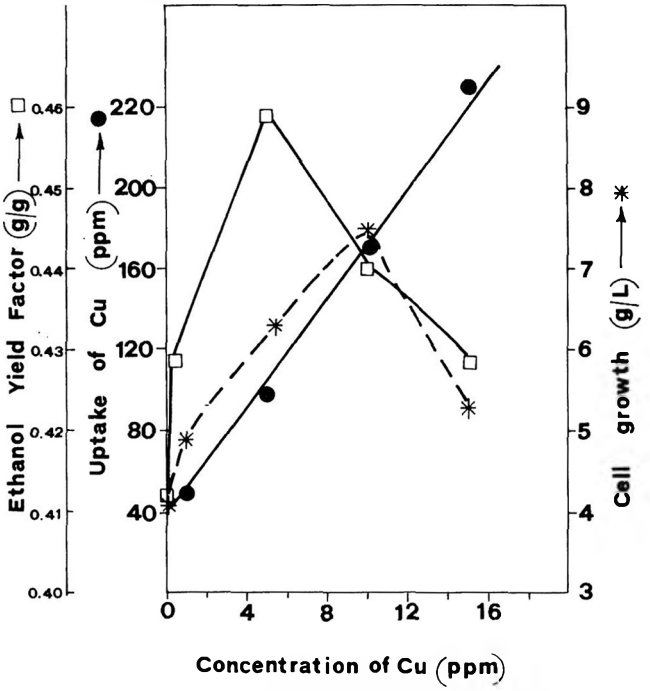

Fig. 4—Cell growth and ethanol yield factor in synthetic fermentation media containing sucrose, as related to uptake of Cu by Saccharomyces cerevisiae cells.

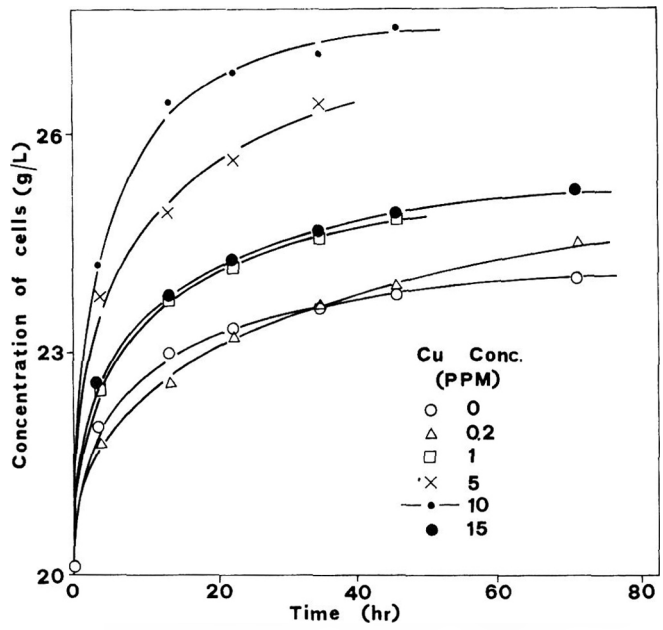

Fig. 5—Cell growth during the fermentation of sucrose at various copper levels.

chumena extract (copper sulfate about 50 ppm) was attemped. The results are summarized in Table 1, Fig. 2 and 3.

Figure 2 shows the ethanol production rate was reduced from batch-to-batch. The first batch in presence of 5 ppm copper was faster than those in presence of 50 ppm. This is paralleled by reduction in rate of cell growth, as clearly shown by the data in Fig. 3. Specifically, the rate of cell growth decreased as the copper content increased from 5 to 50 ppm. The reduction was greater from batch-to-batch with 50 ppm copper. Results in Table 1 revealed the increase of fermentation time, followed by a decrease of ethanol productivity, yield and cell growth from batch-to-batch, were directly related to the increase in copper uptake.

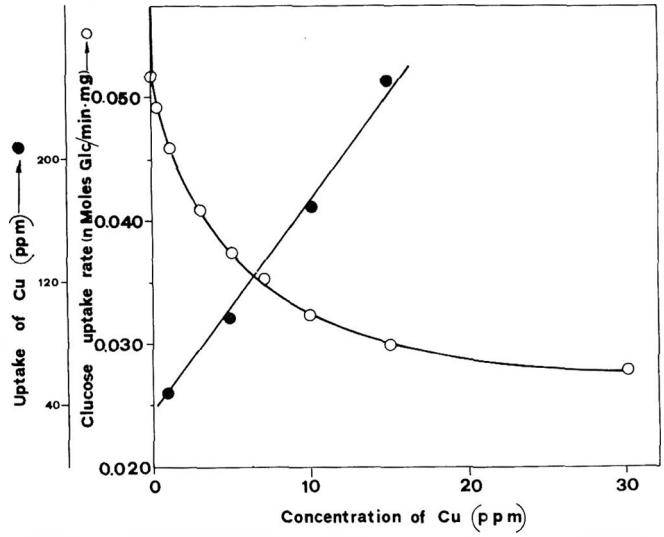

Fig. 6—Glucose uptake rate by Saccharomyces cerevisiae cells, as related to uptake of Cu.

To confirm the observation of the reduction of yield and cell growth with the increase of the uptake of copper, fermentations of synthetic media, containing sucrose, in presence of various copper concentrations (0, 1, 5, 10, 15 ppm) were attemped. The results in Fig. 4, indicate the direct relation between the concentration of copper, and its uptake by the cells. When the uptake of copper was above 100 ppm to 180 ppm the ethanol yield and cell growth diminished sharply. Likewise, the increase of the fermentation time was related to increase in copper uptake. This was measured as increased copper content of synthetic media containing sucrose (Akrida-Demertzi et al., 1988). The reduction in rate of cell growth observed with increase in the copper concentration in raisin extract (Fig. 3) was also confirmed by the synthetic media fermentations (Fig. 5). This figure indicates that at a concentration of 15 ppm copper the rate of cell growth diminished in comparison with those at 5 and 10 ppm.

Finally, Fig. 6 shows that as the concentration of copper increased the glucose uptake rate by cells decreased. The decrease of glucose uptake directly coincides with the increase of copper uptake.

DISCUSSION

IN POTABLE ALCOHOL production raw materials cause problems in fermentations and ethanol production rate is reduced. According to producers this is due to fermentability of the raw material. On the basis of experience of 4-years working in the alcohol production industry, differences are observed in alcohol productivity between various kinds of raw materials, such as molasses, raisins and figs. Observation indicates molasses has lower fermentability than raisins and figs. Moreover, in order to ferment molasses with less problems, at least 25% raisin extracts (from *Trechumena, Piotiki dialogi* and *Chondrada*) are added. This is in agreement with the fact that addition of raisin extracts at less than 6% v/v promoted the fermentation, with *Z. mobilis*, of a synthetic medium containing glucose (Koutinas et al., 1986). This explains why in molasses fermentations by the cell recycle method no more than 15 repeated batch fermentations could be achieved, while in the case of raisin extracts the number reached 300 (Akrida-Demirtzi et al., 1988). Likewise, differences have been observed between sugar beet and sugar cane molasses as well as various kinds of raisins. With raisins, the inhibition was found to be with *Psila* which contains higher copper content (Akrida-Demertzi et al., 1988), and is common in repeated batch fermentations. This necessitated the examination of repeated batch

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Moisture Sorption Isotherms of Dried Apricot, Fig and Raisin at 20°C and 36°C

EROL AYRANCI, GÜLER AYRANCI and ZAFER DOĞANTAN

ABSTRACT

Isotherms were found to be of type I, typical of high sugar foods. Study on temperature dependence of isotherms showed that above water activities of 0.6, moisture content was higher at higher temperature. This was explained by endothermic dissolution of sugar at high water activities. Iglesias and Chirife, Halsey, BET and GAB equations were tested to fit the data. Monolayer moisture contents for the three foods were determined from BET and GAB equations. In both models, monolayer moisture was found to decrease with increasing temperature. Isosteric heat of sorption data from the isotherms at two temperatures, revealed that strength of interactions between water vapor and adsorbent increased with moisture content.

INTRODUCTION

MOISTURE SORPTION ISOTHERM is an extremely important tool in food science because it can be used to predict changes in food stability and to select appropriate package materials and ingredients. An increasing number of works have been reported in this area during the last decade. These included the sorption isotherms of certain foods (Bolin 1980, Ajibola 1986), the temperature dependence of isotherms and determination of heat of sorption (Labuza *et al.*, 1985, Iglesias *et al.*, 1986), and mathematical formulations to represent sorption isotherms (Asbi and Baianu 1986, Chirife *et al.*, 1983). Works on the relation of sorption isotherms to food deterioration reactions have also been included (Rockland and Nishi, 1980).

The purpose of our study was to determine the moisture sorption isotherms of three sugar containing foods, dried apricot, dried fig and raisin at two temperatures, 20°C and 36°C. We also tested the fit of sorption data to some well-known sorption isotherm equations to help find important parameters such as monolayer moisture content.

MATERIALS & METHODS

Materials

All three food samples were obtained from local supermarkets. Raisins were of seedless type. Dried apricots were from Malatya region of Turkey. Dried figs were from the western part of Turkey.

All chemicals used in the preparation of saturated salt solutions were reagent grade and water used was doubly distilled.

Methods

An isopiestic method was employed for the determination of sorption isotherms (Gal, 1975; Labuza, 1984). Seven saturated salt solutions were prepared corresponding to a range of water activities from 0.06 to 0.98. Each solution was transferred into two separate jars in an amount to occupy a space of about 1cm deep at the bottom. A tripod was also placed in each jar. Samples of dried apricot, dried fig and raisin were thin sliced and dried in a vacuum oven at about 30°C for two days. Then duplicate samples of about 0.5g of the dried products were weighed into small crucibles of aluminum foils and placed on tripods in the jars which were then tightly closed. Each of the two sets of seven jars were kept in ovens at 20°C and 36°C for equilibration of samples. This required about 3 to 4 wk. After equilibration, samples were analyzed for moisture content by the vacuum oven method (Karmas, 1980). The percentage difference in equilibrium moisture contents between the duplicate samples was, on the average, less than 1% when the average of the two was taken as the true value. These average values were used in determination of moisture sorption isotherms.

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RESULTS & DISCUSSION

Isotherms

The water sorption isotherms at 20°C and 36°C are shown in Fig. 1 for dried apricot, in Fig. 2 for dried fig, and in Fig. 3 for raisin. In general, all of the isotherms showed characteristics of type I isotherm (Labuza, 1984). This type isotherm is observed for high sugar foods. At low water activities, water can be adsorbed only to surface -OH sites of crystalline sugar. Therefore, moisture content is low in the low water activity region. At high water activities, dissolution of sugar occurs and crystalline sugar is converted into amorphous sugar (Saltmarch and Labuza, 1980). The amount of water to be adsorbed increases greatly after this transition because of the increase in the number of adsorption sites upon breakage of the crystalline structure of sugar. Since all three food products studied were high sugar foods, isotherm type I described above was observed for all of them as shown in Figures 1,2, and 3.

Temperature dependence of isotherms

In general, moisture content is expected to decrease with increasing temperature at a given water activity. This was observed for all three products in the water activity region below about 0.6 (Fig. 1,2, and 3). However, above this region, the opposite result was observed. For all three products the moisture content was larger at 36°C than at 20°C at the water activities above 0.6. The crossing of 20°C and 36°C isotherms at about the water activity of 0.6 in Fig. 1, 2, and 3 was an

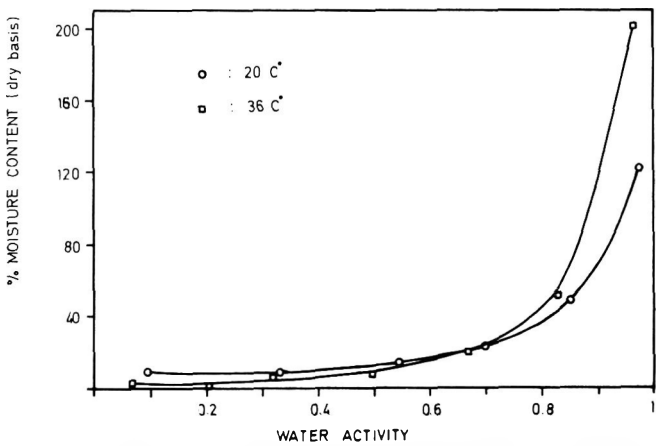

Fig. 1—Moisture sorption isotherms of dried apricot at 20°C and 36°C.

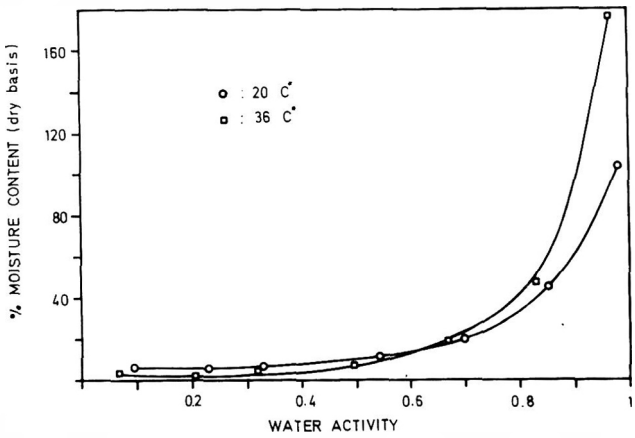

Fig. 2—Moisture sorption isotherms of dried fig at 20°C and 36°C.

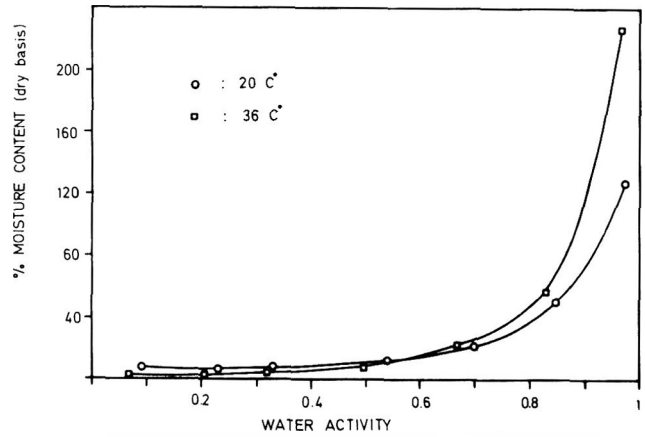

Fig. 3—Moisture sorption isotherms of raisin at 20°C and 36°C.

indication of this variation in moisture content vs. water activity. A similar isotherm crossing behavior was also observed by Saravacos *et al.* (1986) for Sultana raisins.

The unusual temperature effect on isotherms above water activity 0.6 was a result of dissolution of sugar in this water activity range as explained above. Since this process is known to be endothermic, more sugar was being dissolved and thus more water was being held by the food products at higher temperatures.

We concluded from such temperature dependence study that depending on the sugar content and state of sugar in the food, a crossing of isotherms at two different temperatures may be observed similar to those seen in Figures 1,2, and 3. Note that such a crossing may not be observed for fresh fruits since sugar should already be in dissolved form throughout the whole water activity range.

Fitting of sorption data to various isotherm equations

Iglesias and Chirife equation: In the review by Chirife and Iglesias (1978), twenty three equations have been discussed in relation to their validity and applicability to moisture sorption data of foods. Among them, the empirical equation proposed by Iglesias and Chirife was noted to fit the sorption data of high sugar foods. Their equation is given as

$$\ln[M + (M^2 + M_{0.5})^{1/2}] = ba_w + p \qquad (1)$$

where M is the moisture content, a_w is the water activity, $M_{0.5}$ is the moisture content at the water activity of 0.5, b and p are constants. Since the products examined in our study were high sugar foods, the data were treated according to Equation (1). $M_{0.5}$ value was found by linear interpolation between two closest points to the water activity of 0.5 in the isotherm data. Results of the linear regression analysis of the data according to Equation (1) are given in Table 1. The regression coefficients of all seven data points are shown as r_1 in the same table. However, when the data were plotted on a diagram as the left hand side of Equation (1) vs water activity (plot not shown), a better fit could be obtained when only data points at high water activities were considered. Thus, only the last four data points, corresponding to high water activity region, were treated similarly and regression coefficients shown as r_2 (Table 1) were obtained. There was considerable improvement in regression coefficients from r_1 to r_2. This result shows that Iglesias and Chirife Equation represents the isotherms of dried apricot, dried fig and raisin better above the water activity of about 0.5. Therefore, Iglesias and Chirife Equation can be considered to be more successfully applicable to high sugar foods in which sugar is in the dissolved state. Iglesias and Chirife tested their equation (Equation (1)) for sorption data of nine high sugar foods which were mostly fresh fruits with sugar mainly in dissolved form.

Table 1—Analysis of sorption data according to Equation (1)

Product	T/°C	$M_{0.5}$[a]	b[b]	p[b]	r_1[c]	r_2[c]
Dried apricot	20	12.92	4.88	0.52	0.9294	0.9828
	36	8.07	5.60	0.17	0.9461	0.9841
Dried fig	20	10.39	5.06	0.28	0.9500	0.9905
	36	7.87	6.49	−0.58	0.9441	0.9898
Raisin	20	12.24	5.20	0.31	0.9351	0.9847
	36	8.42	6.84	−0.68	0.9560	0.9904

[a] $M_{0.5}$ is in g water/100g dry solid.
[b] b and p values are for regression analysis of the last four data points corresponding to high water activity region.
[c] Regression coefficients obtained when all (r_1) and only the last four (r_2) data points were used.

Table 2—Analysis of sorption data according to Halsey equation

Product	T/°C	Constants of Eq. (2) r	Constants of Eq. (2) c	Regression coefficient
Dried apricot	20	0.666	11.58	0.9820
	36	1.171	5.66	0.9667
Dried fig	20	0.726	8.75	0.9821
	36	1.130	5.75	0.9595
Raisin	20	0.709	10.64	0.9830
	36	1.111	6.70	0.9849

Halsey equation. Halsey equation is also widely used to represent the sorption isotherms of foods (Chirife and Iglesias, 1978). Their equation may be written in the linear form as follows:

$$\ln M = (1/r)\ln c - (1/r)\ln[\ln(1/a_w)] \qquad (2)$$

where c and r are constants, M is moisture content and a_w is the water activity. When the sorption data were treated according to Equation (2), the constants and linear regression coefficients obtained are shown in Table 2. The fit of the data to Halsey equation was quite good as seen from the regression coefficients. Note that in contrast to the treatments according to Iglesias and Chirife equation discussed above, or BET equation discussed below all data points were used with the Halsey equation and quite good fits were obtained in all cases (Table 2).

BET and GAB equations. Until recently, one of the most popular food isotherm equations was the BET equation (Brunauer *et al.*, 1938). This equation provides the value of monolayer moisture content, which is an important parameter in food deterioration studies. However, the BET model is known to hold for water activities up to about 0.5 (Chirife and Iglesias 1978). Even in this low water activity region, the fit of the sorption data to BET equation is not usually very satisfactory.

In recent years, the most widely accepted model for sorption isotherms has been the GAB (from Guggenheim-Anderson-

Table 3—Analysis of sorption data according to BET and GAB equations

Product	T/°C	BET monolayer (g/g solid)	Constants of GAB equation		
			M_m	C	k
Dried apricot	20	0.0591	0.1005	2.443	0.9433
	36	0.0243	0.0987	1.908	0.9849
Dried fig	20	0.0465	0.1100	1.425	0.9235
	36	0.0235	0.1029	1.338	0.9771
Raisin	20	0.0553	0.0998	3.076	0.9466
	36	0.0322	0.0934	6.061	0.9920

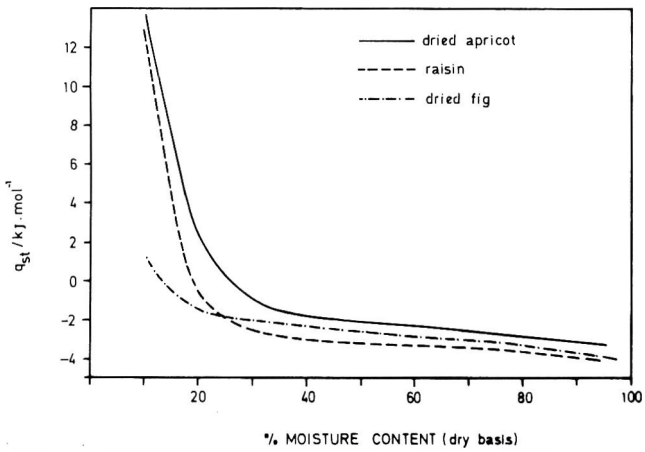

Fig. 4—Variation of the q_{st} term in Eq. (4) with moisture content for dried apricot, dried fig, and raisin.

deBoer) model (Labuza *et al.*, 1985). Treatment of sorption data according to that equation provides not only the value of monolayer moisture content but also other useful information related to heat of sorption of monolayer and multilayer. The equation can be written in the following form:

$$M = M_m Cka_w/[(1 - ka_w)(1 - ka_w + Cka_w)] \quad (3)$$

where M is the equilibrium moisture content, M_m is the monolayer moisture content, a_w is the water activity, C is the Guggenheim constant related to heat of sorption of first layer and k is a factor related to the total heat of sorption of multilayer. The application of GAB equation to sorption data is not as easy as the previous equations because of its nonlinearity. A computer program is required for nonlinear regression analysis of the sorption data according to this equation. The program used in our study involved using the sorption data and some initial values for C, k and M_m parameters. Starting from these initial values, the program determines the best values of C, k and M_m which satisfy the equation by carrying out several iterations for values of these parameters. The initial values were obtained by solving the equation for the three parameters with three of the experimental data points. The results of these analyses are given in Table 3 where the M_m values obtained by BET equation are also given for comparison. In the BET treatment only the first four data points were used. The fit of the data to BET equation was quite poor. It should be noted from Table 3 that M_m values obtained by both models decreased with increasing temperature. This was consistent with the conclusion of Iglesias and Chirife (1976). Another point to note from Table 3 is that the M_m values obtained by GAB model were considerably higher than those obtained by BET model. This was consistent with results obtained by Labuza *et al.* (1985).

It was not possible to make direct comparisons between isotherm equations we used to check their representation of sorption data through linear regression coefficients since one of them, GAB, was in nonlinear form. However, when the mean relative deviation modulus defined by Lomauro *et.al.* (1985), was calculated for the three equations using all of the data points, the representation of GAB model was found best for isotherms of dried apricot at 20°C and at 36°C and of raisin at 20°C, Halsey equation was best for isotherms of dried fig at 36°C and raisin at 36°C while Iglesias and Chirife equation was best for the isotherm of dried fig at 20°C.

Heat of sorption

The study of sorption isotherms at least at two different temperatures provides thermodynamic data on isosteric heat of sorption through use of the integrated form of Clausius-Clapeyron equation (Rizvi and Benado, 1984):

$$\ln(a_{w1}/a_{w2}) = -(q_{st}/R)[(T_1/T_2) - (1/T_1)] \quad (4)$$

$$\text{where } q_{st} = Q_{st} - \Delta H_{vap}(H_2O) \quad (5)$$

In these two equations a_{w1} and a_{w2} are the water activities at a given moisture content at temperatures T_1 and T_2, respectively. Q_{st} is the isosteric heat of sorption, $\Delta H_{vap}(H_2O)$ is the enthalpy of vaporization of water and R is the universal gas constant. The important term here is the isosteric heat of sorption, Q_{st}, which is a measure of the interaction between water vapor and the adsorbent food material.

Q_{st} values were obtained at different moisture contents using the isotherms at 20°C and 36°C in Fig. 1,2, and 3. The plots of these values vs. per cent moisture are shown in Fig. 4 for dried apricot, dried fig and raisin. All three products show similar trends in q_{st} vs. per cent moisture content. Since in the temperature range studied, the enthalpy of vaporization of water can be taken as constant (43 kJ.mol^{-1}), the trends seen in Figure 4 also reflect the variation of isosteric heat of sorption, Q_{st}, with per cent moisture content. Isosteric heat of sorption was large at low moisture content (<15%) and then it decreased sharply reaching a plateau above moisture content about 25%. This type of q_{st} vs. per cent moisture content change is in agreement with results of Saravacos *et al.* (1986) for Sultana raisin and with results of Roman *et al.* (1982) for apple. Large endothermic values of isosteric heat of sorption, Q_{st} at low moisture contents for the three foods studied indicate weaker interactions between water vapor and the adsorbent. These interactions become stronger as moisture content increases.

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Water Sorption Isotherms of Raisins, Currants, Figs, Prunes and Apricots

E. TSAMI, D. MARINOS-KOURIS and Z.B. MAROULIS

ABSTRACT

Moisture sorption isotherms of dried fruits [Sultana raisins, Corinthian (black) currants, figs, prunes, and apricots] were determined at 15, 30, 45, and 60°C, using the standard static gravimetric method developed by the European Cooperation Project COST 90. Experimental curves showed an inverse effect of temperature at high moisture content due to high sugar content of dried fruits. The hysteresis between adsorption and desorption was verified experimentally. The GAB equation was used to predict experimental data for water activity range 0–0.95.

INTRODUCTION

ANALYSIS OF PROCESSES affecting physical, biochemical, and microbiological stability of foods, which in turn determine their quality, is largely based on moisture sorption isotherms data of the materials involved. These isotherms also reveal information about the sorption mechanism and interaction of food biopolymers with water. Finally, they are important in design and optimization of unit operations such as preservation, drying, storing, packaging, and mixing.

Formulation of an analytical relation for prediction of sorption behavior of foods presents difficulties due to complexity of their structure. The addition or removal of water changes the composition and size of the material and can cause phase transitions (dissolution, sugar crystallization, etc.). The change in moisture content as a function of water activity (a_w), is determined by a combination of factors, each of which is predominant in a specific region of the isotherm (Iglesias and Chirife, 1976).

Several researchers have reported sorption data for products and temperatures. Iglesias and Chirife (1975) determined the sorption isotherms of sugar beet root. Bolin (1980) measured ad- and desorption isotherms for California raisins at 25°C. Roman et al. (1982) measured the desorption isotherms of apples in the temperature range 20–60°C, for analysis of thermodynamic properties. Weisser et al. (1982) determined sorption isotherms of sugar substitutes (sorbitol, mannitol, xylitol and palatinit) between 25 and 80°C, and Mazza (1984) determined the sorption isotherms of sugars. Vagenas et al. (1986) reported the adsorption isotherms of Sultana raisins at 30°C, and Saravacos et al. (1986) measured the adsorption isotherms of Sultana raisins in the temperature range 20-35°C. Abdelhag and Labuza (1987) reported results for apricots, and Maroulis et al. (1988) presented data for raisins, figs, prunes, and apricots.

The objective of our study was to provide complete and reliable experimental data for sorption characteristics of some dried fruits, and to suggest a mathematical model for prediction of their sorption behavior as a function of temperature and composition. Five different fruits, namely Corinthian (black) currants, Sultana raisins, figs, prunes, and apricots were used for experimental measurements of ad- and desorption isotherms in the range 15–60°C.

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MATERIALS & METHODS

Materials

All dried fruits used for the experiment were produced in Greece and supplied for this study by the local market. They are characterized by high sugar content and very good organoleptic properties. Fruits used were: Raisins of 13-15% moisture, 82.3%d.b. sugars; Currants of 13-16% moisture, 79.5%d.b. sugars; Figs of ≈ 24% moisture, 75.2%d.b. sugars; Prunes of ≈ 23% moisture, 54.5%d.b. sugars; and Apricots of ≈ 22% moisture, 51.5%d.b. sugars.

Apparatus

The method for determination of sorption isotherms was the standard static gravimetric method developed by the European Cooperation Project COST 90 (Spiess and Wolf, 1983). The experimental apparatus consisted of four thermostatically controlled water baths accurate to ±0.2°C, each containing ten 1-L air-tight glass jars (hygrostats). Each hygrostat contained a saturated salt solution selected to provide defined constant relative humidity inside the jar (Greenspan, 1977). Samples were placed on Petri dishes above the saturated salt solution. Bath temperature was maintained by a Haake F3 thermostat. For low temperatures, a refrigeration unit (Townson and Mercer) was used in conjuction with the heating unit

The accuracy and reliability of the experimental apparatus were checked by measuring the sorption isotherm of standard method reference material MCC (microcrystalline cellulose, Merck Avicel PH101). The mean value of five measurements at ten water activities differed from reference values by not more than the critical difference 0.498 kg water/100kg dry solids. Moreover the standard experimental error was less than the critical value 0.060 kg water/100kg dry solids (Wolf et al., 1984).

Experimental procedure

A representative sample was taken from the bulk of the product and the fruits were cut in thin slices of 1–2 mm (Vagenas et al., 1986). These were placed in 25x25 mm weighing bottles and predried (for measurement of adsorption isotherm) in decanters containing P_2O_5 (ca 15 days, Saravacos et al., 1986) at room temperature.

Desorption isotherms were determined on samples hydrated in decanters over distilled water ($a_w = 1$) at room temperature (Weisser, 1985; Benado and Rizvi, 1985). Product hydration without explicit dissolution of sugars was occured in fourteen days (Tsami, 1988). A small amount of thymol was placed in each decanter to prevent fungal activity (Wolf et al., 1985).

When initial moisture of the sample reached the desired value, the sample was distributed in five weighing bottles for each glass jar. Three water baths containing ten jars each were used for each product and temperature (15 replications for each data point). Four temperatures (15, 30, 45, and 60°C) were used.

The required equilibration time was 15 days based on weights in two successive measurements taken at 1-day intervals not exceeding accuracy of the balance (±0.0001g for a Mettler AE160) (Tsami, 1988; Saravacos et al., 1986).

The total time for removal, weighing, and replacing hygrostats was minimized at about 12 sec (30 sec recommended by Cooperative Project). This minimizes atmospheric moisture sorption or desorption during weighing.

Equilibrium moisture content was determined using a vacuum oven at 70°C and 50 Torr 6 hrs (AOAC, 1980).

Regression of the GAB model

A detailed search of the literature (Van den Berg and Bruin, 1981) indicated the most efficient equation for prediction of the sorption

isotherms was the GAB (Guggenheim-Anderson-deBoer) equation. It is a relatively simple model with a small number of parameters which have physical meaning, and can be applied to a high range of water activities (Weisser, 1985; Van den Berg, 1985).

The GAB equation is normally written:

$$X = Xm\ C\ k\ a_w/[(1 - k\ a_w)\ (1 - k\ a_w + C\ K\ a_w)] \quad (1)$$

where, X is moisture content of the material, a_w is water activity, Xm is monolayer moisture content, while C and k are related to temperature effects. That is,

$$C = Co \exp(\Delta Hc/RT) \quad (2)$$

$$k = ko \exp(\Delta Hk/RT) \quad (3)$$

where T is the absolute temperature (K), R is the gas constant and ΔHc, ΔHk are functions of the heat of sorption of water: $\Delta Hc = Hm - Hn$, and $\Delta Hk = Hl - Hn$. Co and ko are constants, Hm and Hn are heats of sorption of mono and multimolecular layers of water, respectively, and Hl is the heat of condensation of water vapor.

The parameters were estimated by fitting the mathematical model to the experimental data using direct nonlinear regression. This procedure presents several advantages over indirect nonlinear regression (Maroulis et al., 1988). Linear regression on the other hand can give highly erroneous results and should be avoided (Schaer and Ruegg, 1984; Maroulis et al., 1988).

The method of direct nonlinear regression estimates the parameters Xm, Co, ΔHc, ko and ΔHk by fitting the equation resulting from substitution of Eq. (2) and (1) in Eq. (3) to all experimental data.

The parameters are estimated by minimization of the residual sum of squares SST (Least Squares Method, Beck and Arnold, 1977):

$$SST = \sum_{i=1}^{N} \sum_{j=1}^{ni} (\psi_{ij} - y_i)^2 \quad (4)$$

where ψ_{ij} is the experimental value of the dependent variable (equilibrium water content) of the jth replicate of the ith experiment, y_i is the predicted value of the model for the ith experiment, n_i is the number of replicates in the ith experiment, and N is the total number of experiments.

The residual sum of squares, SST, consists of the lack of fit sum of squares, SSR, and the pure error sum of squares, SSE: i.e.,

$$SST = SST + SSE \quad (5)$$

where:

$$SSR = \sum_{i=1}^{N} n_i\ (\psi_i - y_i)^2 \quad (6)$$

$$SSE = \sum_{i=1}^{N} \sum_{j=1}^{ni} (\psi_{ij} - \psi_i)^2 \quad (7)$$

$$\text{and } \psi_i = \sum_{j=1}^{ni} \psi_{ij} \quad (8)$$

The standard deviation between experimental and predicted values, S_R, and the standard experimental error, S_E, can then be calculated from the following equations:

$$S_R^2 = SSR/(N - p) \quad (9)$$

$$S_E^2 = SSE/(M - N) \quad (10)$$

$$M = \sum_{i=1}^{N} n_i \quad (11)$$

where p is the number of parameters.

A model is considered acceptable if the standard deviation, S_R, between experimental and predicted values is close to the standard experimental error, S_E.

According to the F-test the model is acceptable when:

$$S_R^2/S_E^2 < F_{1-\alpha}\ (N - p, M - N) \quad (12)$$

where $F_{1-\alpha}(N\text{-}p, M\text{-}N)$ is the value of the F-distribution for N-p and M-N degrees of freedom at significance level $1-\alpha$.

Table 1—Equilibrium moisture content of dried fruits (% dry basis) adsorption data

Temp	Water activity	Raisins	Figs	Prunes	Apricots	Currants
15	0.113	5.7	6.0	5.7	3.6	4.4
	0.234	6.4	6.7	6.4	3.8	4.9
	0.333	7.9	7.4	7.2	4.9	5.4
	0.431	9.8	10.4	9.2	6.0	8.3
	0.559	15.6	14.9	14.5	11.2	13.9
	0.607	19.0	17.8	17.2	14.7	17.0
	0.741	32.2	29.9	31.1	25.7	29.6
	0.756	34.0	32.1	32.3	26.8	31.5
	0.859	57.3	54.7	53.0	49.7	53.0
30	0.113	3.0	3.0	3.1	2.4	2.5
	0.216	3.2	3.6	3.6	2.8	3.1
	0.324	5.2	4.7	5.3	3.7	4.1
	0.432	8.4	8.3	8.2	5.6	6.9
	0.514	12.5	11.7	11.8	8.6	10.8
	0.560	16.0	14.2	14.3	11.3	12.8
	0.691	28.9	25.1	25.2	20.5	23.2
	0.751	38.1	34.0	34.1	28.4	33.3
	0.836	58.5	52.8	53.3	47.1	52.6
45	0.112	2.0	2.0	.9	1.1	1.1
	0.195	3.3	2.8	1.5	1.2	2.3
	0.311	5.0	4.0	3.5	2.1	3.7
	0.432	8.5	7.5	6.7	4.8	6.8
	0.469	10.5	9.2	7.8	5.6	8.3
	0.520	12.3	11.5	9.3	8.0	11.1
	0.640	21.7	20.2	18.4	15.1	20.1
	0.745	35.3	30.9	34.0	29.9	33.6
	0.817	50.0	44.2	43.1	44.5	50.0
60	0.109	1.1	1.1	.4	.2	.5
	0.160	2.3	1.9	1.1	.9	1.0
	0.293	3.4	3.1	2.6	1.4	3.2
	0.432	7.7	6.5	5.4	4.0	6.6
	0.440	7.3	6.9	6.2	4.1	7.2
	0.497	12.5	9.1	8.3	6.0	9.7
	0.580	15.2	12.0	12.3	9.2	15.8
	0.745	33.4	26.9	29.0	25.3	34.3
	0.803	43.3	35.6	38.0	32.3	49.8

Table 2—Equilibrium moisture content of dried fruits (% dry basis) desorption data

Temp	Water activity	Raisins	Figs	Prunes	Apricots	Currants
15	0.113	7.9	7.3	8.7	5.1	7.5
	0.234	8.2	8.5	9.3	5.7	8.2
	0.333	8.3	9.1	9.6	6.6	8.6
	0.432	10.1	10.4	10.3	9.0	10.5
	0.559	15.7	15.3	16.0	12.6	15.6
	0.607	19.1	19.0	19.4	15.6	19.1
	0.741	34.5	35.0	37.3	31.7	36.5
	0.756	38.8	37.5	40.1	34.9	38.4
	0.859	69.8	57.2	69.3	60.7	60.6
30	0.113	4.3	4.7	4.2	4.0	3.6
	0.216	4.7	5.1	4.6	4.8	4.0
	0.324	5.6	5.8	5.7	5.8	4.7
	0.432	9.3	8.5	8.5	7.7	8.0
	0.514	12.8	12.0	12.2	10.5	11.4
	0.560	16.4	14.7	14.4	12.5	13.6
	0.691	28.9	25.3	25.8	23.8	25.8
	0.751	38.6	34.2	35.7	32.3	34.6
	0.836	59.1	52.8	56.1	53.0	55.5
45	0.112	2.2	2.0	1.2	1.6	.9
	0.195	3.3	2.9	1.8	2.2	2.4
	0.311	5.1	4.2	3.7	3.0	3.8
	0.432	8.8	7.5	6.8	5.7	7.1
	0.469	10.5	9.2	7.8	7.0	8.5
	0.520	12.4	11.8	9.6	9.0	11.2
	0.640	21.6	20.8	18.6	18.6	20.3
	0.745	35.7	31.1	35.0	32.4	33.4
	0.817	50.1	44.8	48.8	50.2	52.6
60	0.110	1.0	1.1	.4	.5	.5
	0.160	2.2	2.1	1.2	1.1	1.4
	0.293	3.6	3.2	2.4	1.7	3.2
	0.432	7.3	6.9	5.5	4.1	6.5
	0.440	7.4	7.1	6.3	4.7	7.3
	0.497	10.6	9.2	9.0	6.3	10.1
	0.580	14.9	12.7	12.5	10.7	17.1
	0.745	33.4	28.5	31.5	26.9	33.7
	0.803	43.2	35.6	40.3	35.3	50.3

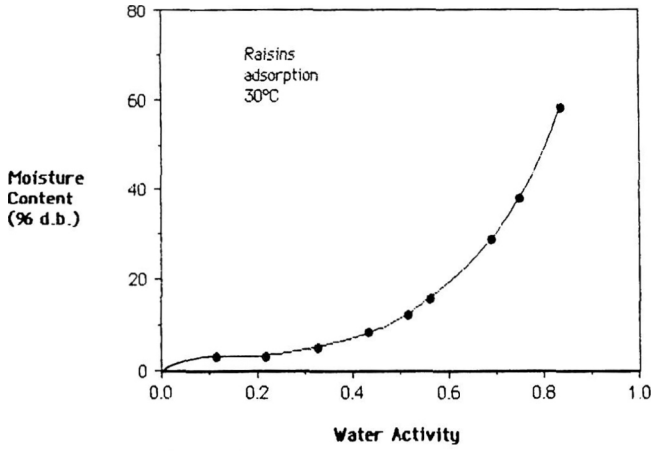

Fig. 1—Adsorption isotherm

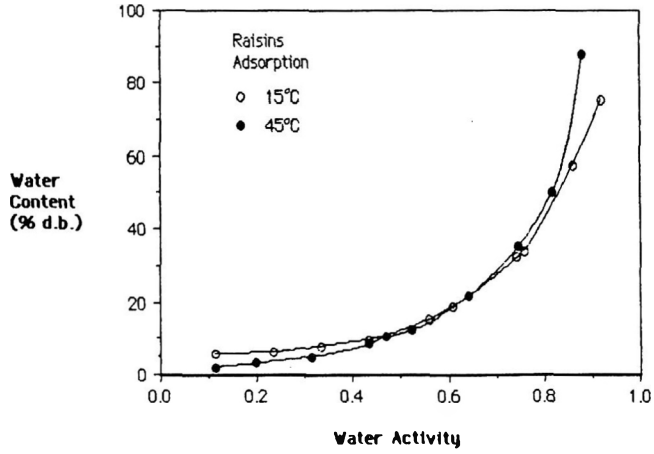

Fig. 2—Effect of temperature on the isotherm.

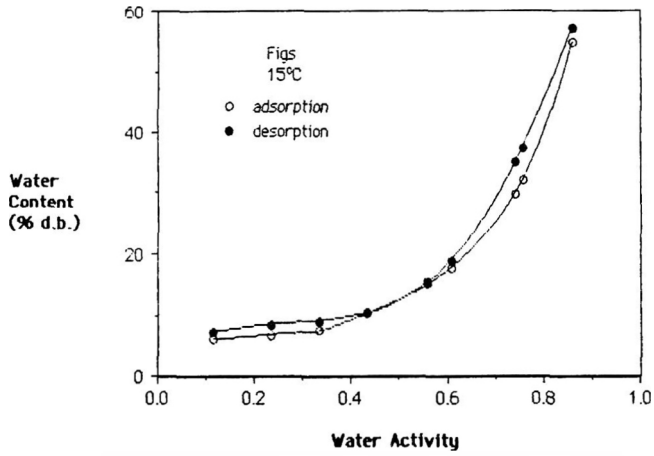

Fig. 3—Hysteresis between adsorption and desorption isotherm at low temperatures.

RESULTS & DISCUSSION

THE EXPERIMENTAL ADSORPTION and desorption isotherms for several temperatures (Tables 1 and 2) report mean equilibrium moisture content at each water activity (a_w).

Figure 1 shows the adsorption isotherm of Sultana raisins. It had the sigmoid shape characteristic of materials with high sugar content (Weisser, 1985). The isotherms for the other fruits were similar.

At low water activities, up to $a_w = 0.1$, there was a steep increase in moisture content, indicating physical sorption on

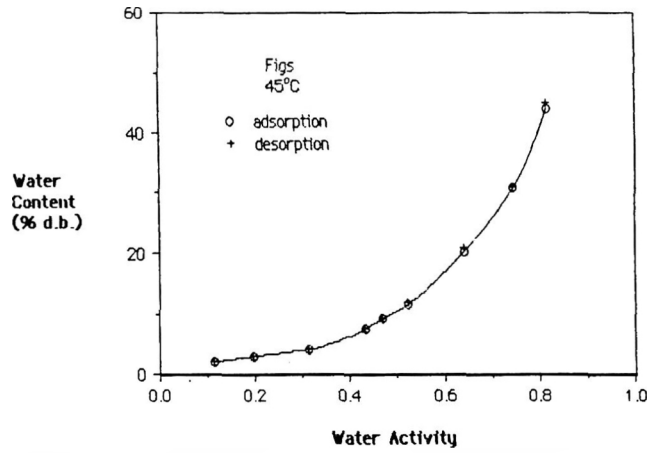

Fig. 4—Hysteresis between adsorption and desorption isotherm at high temperatures.

strongly active sites of the biopolymer. Hellen (1985) calculated the heat of sorption for $a_w < 0.1$ and verified existence of strongly active sites in this range of water activity. Weisser (1985) reported that at low water activities there was a local dissolution of sugar alcohols, a swelling of the biopolymer, and appearance of new active sites.

For intermediate water contents, up to 0.55–0.70 the sorption took place in less active sites (the slope of the curve decreased gradually). From this point on there was gradual dissolution of sugars which resulted in a complete exudation of sugars in solution at high water activities ($a_w > 0.8$).

Effect of temperature

It is widely accepted that an increase in temperature results in decreased equilibrium moisture content (Hill and Rizvi, 1982). This was verified by our results for the dried fruits up to a water activity of about 0.55–0.70. In that region the curves for several temperatures intersect. For water activity of values higher than 0.7 there was inversion of the effect of temperature (i.e. equilibrium moisture content increased with temperature). This was due to an increase in solubility of sugars in water. Figure 2 clearly shows the intersection point and the inverse effect of temperature (between 15 and 45°C) on moisture sorption curves of Sultana raisins.

The intersection (inversion) point depends on the composition of the food and the solubility of sugars (Weisser et al., 1982). For Sultana raisins and currants the inversion point was about $a_w = 0.55$, likewise $a_w = 0.65$ for figs, $a_w = 0.70$ for prunes; and for apricots, possessing the lowest sugar content of the dried fruits tested, at about $a_w = 0.75$ (Abdelhaq and Labuza, 1987).

The effect of temperature on sugar-containing products has also been studied by Saravacos et al. (1986) for Sultana raisins, Weisser et al. (1985) for sugar alcohols, and Hellen and Gilbert (1985) for biscuits, with results similar to ours.

For products with low sugar (e.g. apples) the curves do not intersect (Roman et al., 1982). For products with high protein or high starch content, there is also no intersection point with increase temperature (Benado and Rizvi, 1985).

Husteresis

The adsorption and desorption isotherms exhibited the phenomenon of hysteresis, in which the equilibrium mositure content was higher at a particular water activity for desorption curve than for adsorption. This is shown in Fig. 3 and 4 for dried figs. The hysteresis was significant for $a_w < 0.5$–0.6 and at temperatures 15 and 30°C. Similar results were found for all dried fruits studied.

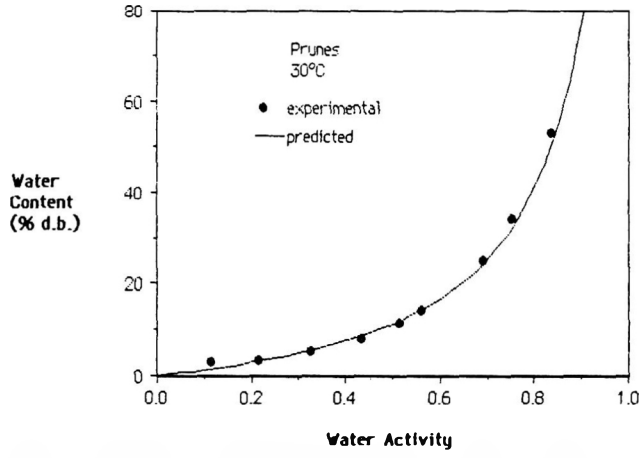

Fig. 5—Experimental and predicted values of an isotherm.

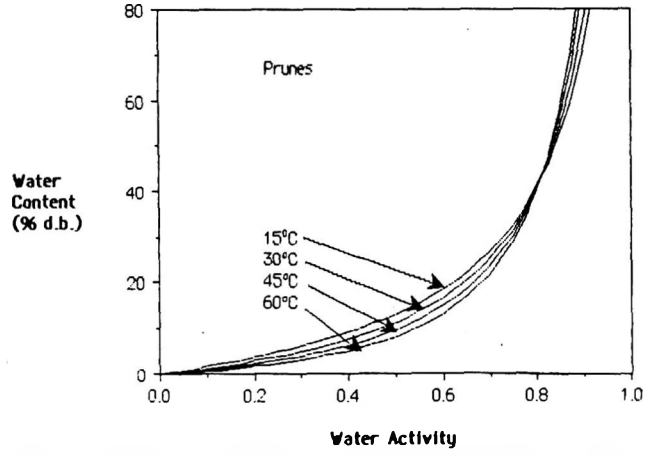

Fig. 6—Adsorption isotherms predicted by the GAB model at several temperatures.

Table 3—Estimated GAB parameters for dried fruits

Dried fruit	Xm	Co	ΔHc	ko	ΔHk	SR	SE
Adsorption							
Raisins	14.0	.01557	10.5	1.30	−0.793	1.71	1.28
Currants	17.3	.05725	5.7	1.58	−1.341	1.16	1.35
Figs	9.7	.00023	22.5	1.22	−0.531	1.48	0.90
Prunes	12.6	.00064	18.5	1.42	−0.994	1.66	0.68
Apricots	11.7	.00186	14.6	1.44	−0.980	1.65	0.71
Desorption							
Raisins	10.9	.00526	14.3	1.06	−0.154	1.62	0.95
Currants	15.0	.00166	15.6	1.60	−1.299	1.70	1.66
Figs	11.8	.00046	20.2	1.11	−0.361	1.67	1.03
Prunes	11.7	.00051	19.6	1.08	−0.227	2.02	2.99
Apricots	10.5	.00015	22.2	1.30	−0.659	1.84	1.36

The water activity below which a significant hysteresis effect was manifested was inversely proportional to the sugar content of the fruits. This is evidently due to the dissolution of the sugars, which is more pronounced at high water activities. Wolf et al. (1972) found hysteresis in dried apples up to a water activity value of 0.65, Chinachoti and Steinberg (1986) in sugar containing starch up to $a_w = 0.6$, and Bolin (1980) in raisins (with very high sugar content) up to $a_w = 0.3$. The absence of hysteresis at 45 and 60°C was due to the dissolution and crystallization of sugars at high temperatures (Roman et al., 1982).

Several theories have been formulated to explain the phenomenon of hysteresis. At present, no theory has given a complete insight into the several mechanisms responsible. The principal factors affecting hysteresis are composition of the product, its temperature, storage time, drying temperature, and the number of successive adsorptions and desorptions. Irreversibility of the ad- and desorption curves, on the other hand, is very significant for the quality characteristics of a food material (Benado and Rizvi, 1985).

Predictive equations

The results of the direct nonlinear regression for the dried fruits are presented in Table 3. The posed criterion for acceptance of the model was fully satisfied. The value of the monomolecular moisture content (Xm) was always less than 10 gH20/100g d.s., which was the maximum value reported for food materials (Van den Berg, 1985; Labuza et al., 1985).

The experimental and predicted adsorption values (using the GAB equation) for prunes at 30°C are shown in Fig. 5. The agreement between experimental and predicted values was quite satisfactory and well within experimental error.

Figure 6 shows the adsorption isotherms of prunes predicted by the GAB equation for several temperatures. The effect of temperature, as well as intersection (inversion) point can be clearly seen.

CONCLUSIONS

THE EXPERIMENTAL MEASUREMENT of adsorption and desorption isotherms of dried fruits at 15, 30, 45 and 60°C using the standard apparatus and method developed by the Cooperation Project COST 90 provided necessary data to fill a gap in the literature. The experimental curves indicated hysteresis and inversion of the effect of temperature. Hysteresis was significant for $a_w < 0.6$ and temperatures up to 30°C, while it was absent for higher water activities and temperatures. The inversion of the effect of temperature took place in the range $a_w = 0.55$–0.70 depending on sugar content. The GAB equation proved successful in fitting experimental data over almost the entire range $a_w = 0$–0.95.

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Polysaccharide Effects on Cross-Flow Microfiltration of Two Red Wines with a Microporous Alumina Membrane

M.-P. BELLEVILLE, J.-M. BRILLOUET, B. TARODO DE LA FUENTE, and M. MOUTOUNET

ABSTRACT

Two red wines, from the same cultivar, differing by polysaccharide content were filtered using cross-flow microfiltration with an alumina membrane, pore size 0.2 μm. Filtration flow rates were different. The wine richest in polysaccharides (A) showed a typical external fouling profile with progressive stabilization, while wine B showed a permanent decrease in flux (external and internal fouling). Retention of polysaccharides negatively correlated with their hydrodynamic volume, overall losses being higher for wine B. Polysaccharides had a possible role in fouling related to their structures and molecular weight distribution in the two wines.

INTRODUCTION

CROSS-FLOW MICROFILTRATION has been intensively studied recently as an attractive process in enology for one-step clarification, sterilization and stabilization of crude wines (Sachs et al., 1983; Poirier et al., 1984; Mietton-Peuchot, 1984; Lüdemann, 1987; Serrano et al., 1988). Microfiltration of musts prior to or during fermentation could lead to new beverages (Barillère et al., 1985). Peri (1987) recently reviewed developments and limitations of tangential cross-flow microfiltration in enology.

A major obstacle to development of microfiltration in enological area is insufficient filtration flow rates (10–150 L/hr/m^2 depending on the filtering device) to get return from investment. Another is alteration of wine quality due to retention of some constituents. Both these obstacles result from fouling of filtrating media.

The purpose of our work was to study the possible role of polysaccharides from wines in fouling cross-flow microfiltration membranes. Two red wines were chosen which differ in polysaccharides and reaction to microfiltration. Retention of their carbohydrate polymers by a microporous mineral membrane was also characterized.

MATERIALS & METHODS

Wine samples

Two samples of wine were prepared by different processes from fully matured grapes of the same cultivar (Carignan noir) harvested in 1987 in the same vineyard of the INRA; Pech-Rough Narbonne Experimental Station (Gruissan, France). Grapes were destemmed, crushed before fermentation (5 days) followed by post-maceration (5 days) (Wine A). Grapes were also destemmed, crushed, rapidly heated at 70°C (Brillouet et al., 1989) before pressing and the must was put to ferment for 5 days (Wine B). Wines were centrifuged at 6,800 g and then stored at ambient temperature untill microfiltration. Preparation of both wines was similarly repeated after 1988 vintage. All other operations were conducted on wines from 1987-88 vintages with essentially the same results in most respects.

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Cross-flow microfiltration

The cross-flow microfiltration experiments were carried out in a laboratory-sized plant from Imeca OEnologie (Clermont l'Hérault, France) equipped with a tubular multichanelled membrane (MEMBRALOX®) from SCT (Tarbes, France). The membrane was porous alumina of a precisely defined average pore size of 0.2 μm, total filtration area 0.18 m^2. More details about nature, structure and properties of the membrane were reported by Gillot and Garcera (1984). Operating conditions were used according to Poirier et al. (1984): crossmembrane pressure (from inside to outside) 3 bar; linear flow velocity 4.5 m/sec; temperature 20°C. Each wine (35 L) was filtered, the filtrate being permanently recirculated into the crude wine for 60 min filtration before starting effective filtration without recirculation (see Fig. 1). Between filtrations, the membrane was thoroughly washed 2x with 2% sodium hydroxide then 2% nitric acid at 80°C, then the flow rate was adjusted with water to 1,500 L/hr/m^2 under 1 bar.

Isolation of proteins and polysaccharides from wines and filtrates

Wine aliquots (750 mL) were extensively dialyzed against distilled water 2 days at ambient temperature than an additional 3 days in the cold. After 2x vacuum concentration at 40°C, 200 mL were injected onto a Sephadex LH 20 column (92 x 2.6 cm; Vo = 170 mL) and eluted with 0.2M sodium chloride at 120 mL/hr. Fractions were collected and analysed for protein and polysaccharide as described below. Fractions containing both proteins and carbohydrate were pooled, dialyzed against distilled water, concentrated and freeze-dried (discolourized colloids).

Analyses

Filtrability Index (Vmax). Vmax was estimated by filtrating wine under pressure (1 bar) on a Millipore membrane (diameter 25 mm; pore size 0.65 μm). Volume of filtered wine was measured after 2

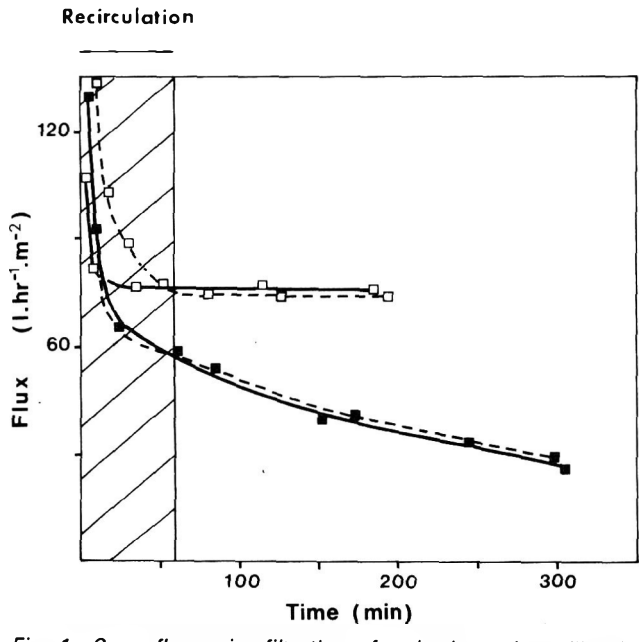

Fig. 1—Cross-flow microfiltration of red wines. Area filtration flow rate as function of time. (□———□) wine A (1987); (□- -□) wine A (1988); (■———■) wine B (1987); (■- -■) wine B (1988).

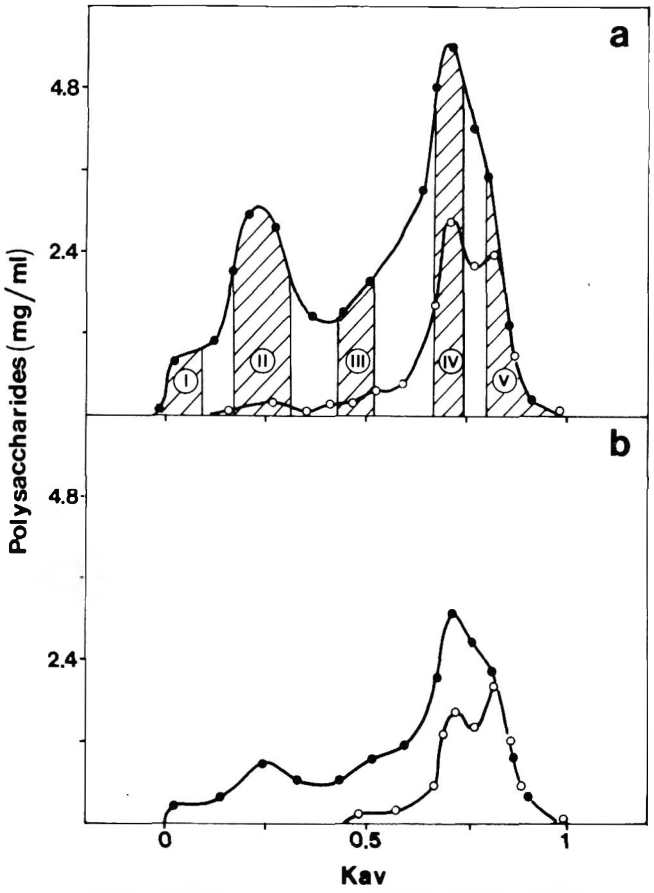

Fig. 2—Gel Permeation chromatography of soluble polysaccharides from wine A (vintage 1987): (a) crude wine; (b) filtered wine) on a column of Ultrogel AcA 34. (●———●) neutral sugars; (○———○) uronic acids.

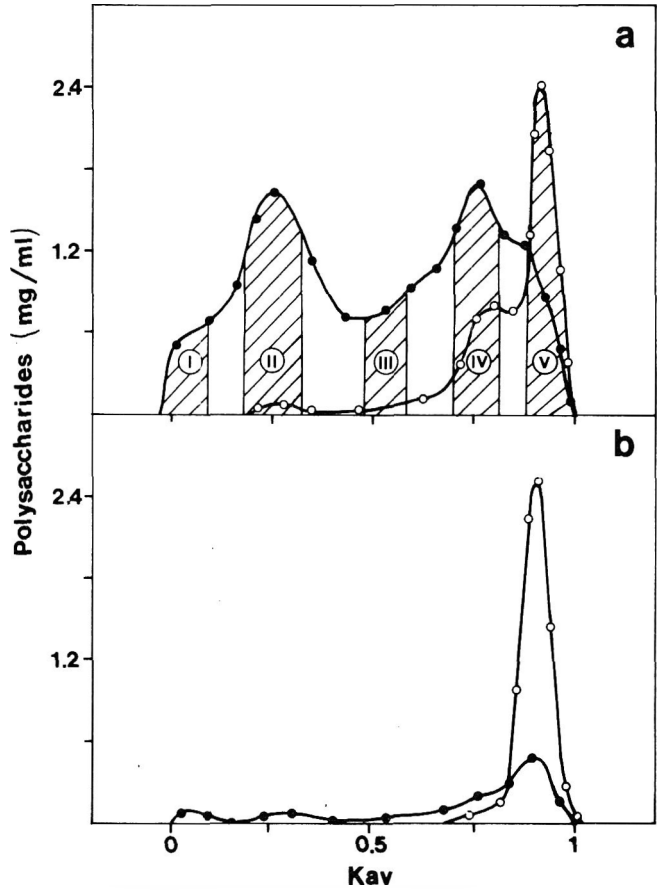

Fig. 3—Gel permeation chromatography of soluble polysaccharides from wine B (vintage 1987): (a) crude wine; (b) filtered wine on a column of Ultrogel AcA 34. (●———●) neutral sugars; (○———○) uronic acids.

Table 1—Filtrability index (Vmax), turbidity and color indexes of crude and filtered wines A and B (vintage 1987)

	Wine A		Wine B	
	Crude	Filtered	Crude	Filtered
Vmax (mL)	169	6800	155	14105
Turbidity (abs 650 nm)	0.295	0.210	0.348	0.138
Absorbance				
420 nm	0.502	0.391	0.454	0.221
520 nm	0.735	0.658	0.597	0.293
620 nm	0.163	0.118	0.153	0.061
Color intensity index	1.40	1.17	1.21	0.58
Hue	0.68	0.59	0.76	0.75

min (V_1) and 5 min (V_2). Vmax was calculated as follows: $Vmax = 3/(5/V_2 - 2/V_1)$. (Gaillard, 1977).

Turbidity and color. Turbidity was estimated by measuring absorbance at 650 nm. Color was determined by direct colorimetric reading at 420, 520, and 620 nm. Results were expressed as color intensity index (Σ abs 420, abs 520, abs 620 nm) and hue (abs 520 nm/abs 420 nm)

Proteins. Protein content was estimated by the method of Lowry et al. (1951) as modified by Potty (1969).

Polysaccharides. Galacturonic acid and neutral sugars were measured by automated *m*-phenylphenol (Blumenkrantz and Asboe-Hansen, 1973; Thibault, 1979) and orcinol (Tollier and Robin, 1979) methods respectively, responses being corrected for mutual interferences. Neutral monosaccharides were released from wine polysaccharides by hydrolysis with 2M trifluoroacetic acid (1 hr 15 min; 120°C) and analysed by gas-liquid chromatography (Saulnier et al., 1988 a) as their alditol acetate derivatives (Albersheim et al., 1967).

Structure of polysaccharides was obtained by methylating colloids (2 mg) with methyl iodide according to Hakomori (1964) as described by Jansson et al. (1976) and Saulnier et al. (1988 a). After hydrolysis, partially methylated sugars were derivatized into alditol acetates as described.

Table 2—Macromolecular constituents (polysaccharides and proteins) of crude and filtered wines A and B (vintage 1987)[a]

	Wine A		Wine B	
	Crude	Filtered	Crude	Filtered
Discolorized colloids	929	443	545	140
Proteins	63	36	50	14
Polysaccharides	683	314	356	88
Acidic	158	80	90	61
Neutral	525	234	266	27
Rhamnose	40	29	20	6
Fucose	4	2	2	2
Arabinose	199	95	74	6
Xylose	4	2	2	1
Mannose	126	48	85	5
Galactose	136	49	72	4
Glucose	16	9	11	3

[a] All data in mg/L of wine.

Fractionation of wine colloids by gel permeation chromatography

Aqueous solutions (2 mL) of discolourized colloids (10 mg) were injected onto an Ultrogel AcA 34 (IBF, France) column (90 x 2.2 cm; Vo = 120mL, Vt = 336mL) and eluted with 0.05M acetate buffer (pH 4.8) containing 0.3M sodium chloride at 34 mL/hr. Fractions (5.7 mL) were collected and analysed for galacturonic acid and neutral sugars. Colloids were also separated on a Sephadex G75 Superfine (Pharmacia Fine Chemicals, Sweden) column (90 x 1.6 cm; Vo = 65 mL, Vt = 190 mL) with 0.07M phosphate buffer (pH 7.0) at 10 mL/hr. Fractions (2.5 mL) were collected and analyzed for galacturonic acid, neutral sugars and protein.

RESULTS & DISCUSSION

Filtration flow rates

Filtration profiles for wines A and B are shown in Fig. 1. Wine A exhibited a filtration pattern agreeing with the theo-

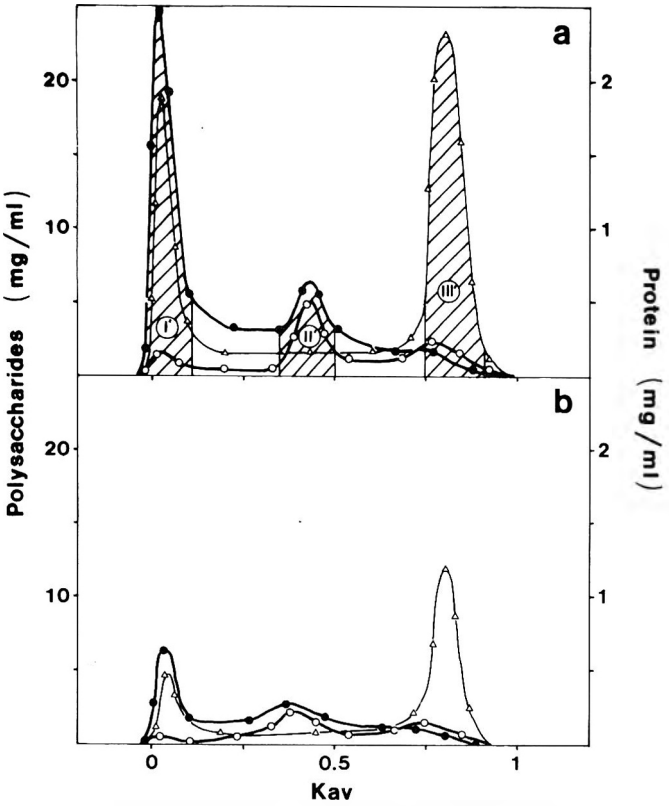

Fig. 4—Gel permeation chromatography of soluble polysaccharides from wine A (vintage 1987): (a) crude wine; (b) filtered wine on a column of Sephadex G75. (●———●) neutral sugars; (○———○) uronic acids; (△———△) proteins.

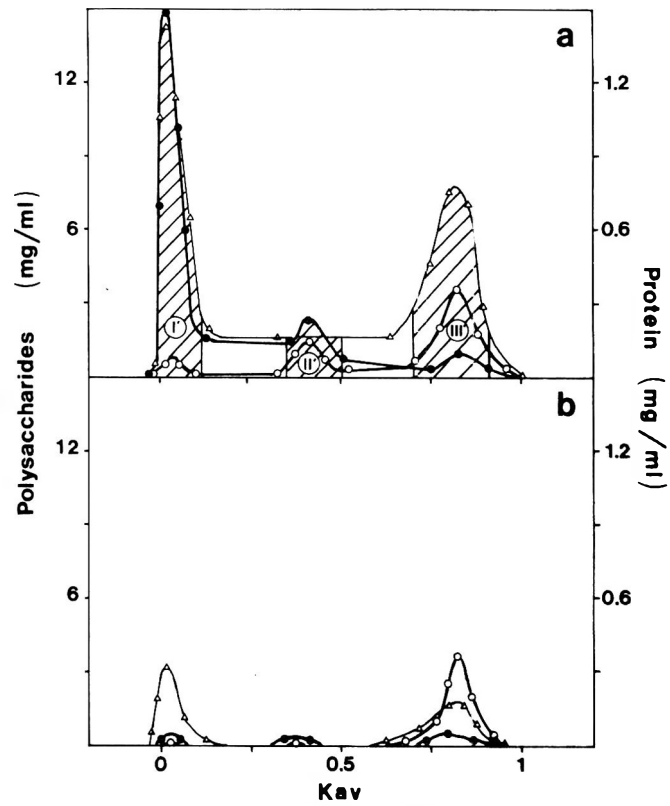

Fig. 5—Gel permeation chromatography of soluble polysaccharides from wine B (vintage 1987): (a) crude wine; (b) filtered wine on a column of Sephadex G75. (●———●) neutral sugars; (○———○) uronic acids; (△———△) proteins.

retical model of Bennasar and Tarodo de la Fuente (1987). Flow rate decreased sharply during the first minutes of filtration, this drop corresponding to formation of the concentration polarization layer and deposition of some material at the surface of the membrane (external fouling), then remained stable (76 L/hr/m^2) up to the end of filtration (t = 190 min) when the volumetric concentration factor, (V.C.F. = volume of wine submitted to filtration residual volume of unfiltered wine) reached 5.4. Wine B behaved differently upon cross-flow microfiltration since after the initial sharp drop, the flow rate did not stabilize and continued to decrease to the end of the experiment (t = 315 min; V.C.F. 5.8). This profile could be due to inner fouling of the membrane superimposed on the external fouling (Bennasar and Tarodo de la Fuente, 1987). An important point is that almost identical filtration profiles were obtained for both vintages of wine A (1987-88) and for both of wine B (1987-88), indicating the observed permeation curves are closely related to the wine making scheme, and not particularly to the year of vintage.

Characterization of wines and filtrates

Both wines A and B showed similar filtration with close Vmax(Table 1), wine B being slightly more turbid than wine A. For both wines Vmax dramatically increased after microfiltration, far past the critical threshold value on which wines could be passed on cartridges prior to final bottling (Gaillard, 1984). However filtered wine B had a Vmax double that of wine A.

A major decrease in color indexes was observed after filtration of wine B which turned from purplish red to rosé. The percent relative distribution of various color constituents was unaltered by microfiltration indicating that no differential retention or oxidative alteration of color components occured. Color reduction was much less with wine A.

Wine A contained about double the discolourized colloids (total soluble proteins, polysaccharides, and residual polyphenolics) of wine B (Table 2), an unexpected finding considering filtration flow rates (Fig. 1). Concentration of both wines in carbohydrate polymers were proportional to colloids, wine A being two times richer than wine B, and (neutral/acidic) sugar ratios were comparable. The neutral sugar distribution of polysaccharides from the two wines was analogous, wine A being however richer in arabinose than wine B; predominance of mannose, galactose and arabinose as constituent monosaccharides of wine carbohydrate colloids has been reported (Brillouet et al., 1989). Soluble proteins were comparable in both wines and 6 to 10x less than polysaccharides as usually observed (Feuillat, 1987).

Microfiltration of wines A and B lowered their colloids by 52 and 74%, proteins by 43 and 72%, and polysaccharide by 54 and 75%, respectively. Compared with permeation flow rates (Fig. 1), wine B which exhibited a permanent decrease and a final flow rate 2x lower than wine A, also had a strongly reduced colloidal spectrum. Moreover 90% of neutral polysaccharides were retained after filtration of wine B compared to 57% for wine A. Acidic polymers were less affected in wine B (32% retention) than in A (49%). The relative neutral sugar distribution was also strongly altered in filtrate from wine B, rhamnose, xylose, and glucose being concentrated. More minor variations were noted after filtration of wine A, except for rhamnose relative content. In both wines, arabinose was little increased as compared to galactose and mannose.

Gel permeation chromatography of colloids from wines and filtrates

When colloids from both wines and corresponding filtrates were separated on Ultrogel with simultaneous colorimetric

Table 3—Methylation analysis of polysaccharide fractions from crude wines A and B and their corresponding filtrates after separation by GPC

Methyl ether	Linkage	Crude wine A					Crude Wine B				
		I[a]	II[a]	III[a]	II'[b]	III'[b]	I[a]	II[a]	III[a]	II'[b]	III'[b]
2,3,4-Me_3-rha[c]	L-Rha*p*-(1	29[d]	61	19	40	14	30	75	29	41	15
3,4-Me_2-rha	2)-L-Rha*p*-(1	40	22	29	37	71	50	10	33	31	70
3-Me-rha	2,4)-L-Rha*p*-(1	31	17	52	23	15	20	15	38	12	10
rha	2,3,4)-L-Rha*p*-(1									16	5
Total		1[e](1)[f]	2(1)	4(4)	16(18)	41(38)	2	2	3	20(15)	49(40)
2,3,5-Me_3-ara	L-Ara*f*-(1	55	89	46	38	44	82	88	43	64	79
2,5-Me_2-ara	3)-L-Ara*f*-(1				3	4	1	1	2	2	4
3,5-Me_2-ara	2)-L-Ara*f*-(1		1	1	1	2	1	2	2	2	
2,3-Me_2-ara	5)-L-Ara*f*-(1	28	5	28	35	39	14	7	30	26	17
2-Me-ara	3,5)-L-Ara*f*-(1	17	5	25	23	11	2	2	23	6	
Total		5(1)	26(21)	44(36)	51(50)	26(29)	12	25	31	43(37)	16(15)
2,3,4,6-Me_4-gal	D-Gal*p*-(1	9	7	12	25	27	4	4	5	26	28
2,4,6-Me_3-gal	3)-D-Gal*p*-(1	56	21	26	10	9	22	19	21	14	8
2,3,4-Me_3-gal	6)-D-Gal*p*-(1	4	6	8	6	4	7	7	10	8	2
2,3,6-Me_3-gal	4)-D-Gal*p*-(1	5	1	3		19	2	2		3	30
2,6-Me_2-gal	3,4)-D-Gal*p*-(1	5	6	10	22	29	6	6	8	11	19
2,3-Me_2-gal	4,6)-D-Gal*p*-(1		2	2	6	2		1	3	4	4
2,4-Me_2-gal	3,6)-D-Gal*p*-(1	16	42	29	11	4	45	47	41	17	5
2-Me-gal	3,4,6)-D-Gal*p*-(1	5	15	10	20	6	14	14	12	17	4
Total		12(13)	47(34)	20(20)	22(21)	24(23)	21	43	23	25(29)	26(20)
2,3,4,6-Me_4-man	D-Man*p*-(1	32	32	31	11	28	32	32	30	27	26
3,4,6-Me_3-man	2)-D-Man*p*-(1	21	19	33	69	48	22	19	31	52	51
2,4,6-Me_3-man	3)-D-Man*p*-(1	25	24	21	9	15	23	25	20	16	5
3,4-Me_2-man	2,6)-D-Man*p*-(1	22	25	15	11	9	23	24	19	5	18
Total		82(85)	25(44)	32(40)	11(11)	9(10)	65	30	43	12(19)	9(25)

[a] Fractions from gel permeation on Ultrogel AcA 34.
[b] Fractions from gel permeation on Sephadex G 75,
[c] 2,3,4-Me_3-rha = 2,3,4-tri-0-methyl-1,5-di-0-acetyl-rhamnitol, etc.
[d] Relative mole percent of rhamnose methyl ethers within the rhamnose family, etc.
[e] Relative mole percent of each parent sugar family (sum of ethers from one sugar type) within total sugars.
[f] Same as (e) for filtered wine.

analyses of neutral and acidic polysaccharides, crude wines A (Fig. 2a) and B (Fig. 3a) exhibited polysaccharide material throughout the fractionation range. Five fractions (I→V) with constant (neutral/acidic) sugar ratios were detected at respective Kav 0.05, 0.24, 0.46, 0.70, and 0.82. Fractions II and IV represented the bulk of neutral polymers in wines A and B as reported by Brillouet et al. (1989). Fraction IV was however predominant in wine A. Acidic moieties were eluted in the high Kav range, fractions IV and V concentrating more than 85% of total injected uronic acids. Fraction V was preponderant in wine B as compared to wine A. These acidic fractions are expected to arise from native grape pectins (Saulnier et al., 1988a) through depolymerization by endogenous pectinases. Different balances between IV and V in the two wines reflect different conditions in the two processes.

Colloids were also separated on Sephadex G75 (Fig. 4a and 5a) into three populations I', II', and III' eluted respectively at Kav 0, 0.4 and 0.8. Comparison of chromatograms from Ultrogel AcA 34 and Sephadex G75 showed that fraction I' included weakly acidic fractions (I→III) from Ultrogel and that II' and III' corresponded respectively to IV and V which were not properly separated on Ultrogel. These were then submitted to methylation structural analysis (Table 3). Proteins were also determined in both crude wines. Two fractions co-eluted respectively with the carbohydrate fractions I' (MW > 70,000) and III' (5,000 < MW < 12,000)(Figs. 4a and 5a). Further characterization of these protein fractions are reported elsewhere (Brillouet et al., 1990 b).

Cross-flow microfiltration affected the relative distribution of soluble polysaccharides from wine A (Fig. 2b), and even more for wine B (Fig. 3b). Losses in neutral and acid polymers negatively correlated to their hydrodynamic volume, the bulkiest molecules being more retained than small ones. The following figures were calculated from the chromatograms: wine A (neutral sugars) I (74%), II (69%), III (60%), IV (50%), V (34%) - (acidic sugars) II (72%), III (58%), IV (47%), V (13%); wine B (neutral sugars) I→IV (>90%), V (61%) - (acidic sugars) II→IV (≈ 100%), V (6%). Microfiltration of the two wines resulted in a relative decrease in proteins of filtrates eluted in the high molecular range (Fig. 4b and 5b).

Structure of polysaccharides from wines and filtrates

Various polysaccharide fractions from both wines and their related filtrates after separation by gel permeation on Ultrogel or Sephadex were methylated with methyl iodide and results are shown in Table 3.

Methylation showed that, in agreement with compositional analysis of carbohydrate colloids (Table 2), arabinose, galactose and mannose were the main sugar constituents. Fucose and xylose were in trace amounts in all fractions. Glucose methyl ethers were omitted since, after discolorization on Sephadex LH 20, some fractions were still faintly colored in light purple. Thus part of glucose might have arisen from residual glucosylated anthocyanins. The relative proportions of constituent sugars were almost the same in the major fraction II from both wines while differences were observed in others. Fractions III, II' and III' were proportionnally higher in arabinose in wine A as compared to wine B which confirmed the greater amount of this sugar in wine A (Table 2).

The distribution of sugar methyl ethers was complex in all fractions as reported (Brillouet et al., 1989). Although each fraction corresponded to mixtures of polysaccharides originating from both grape and yeast, the presence of some typical ethers allowed us to refer to known structures. Fraction II, exhibiting the same overall distribution in both wines, had arabinose essentially under the terminal non-reducing position and galactose mainly as 3,6-,3- and 3,4,6-linked moieties. These structural features correspond to type II arabino-3,6-galactans previously isolated from grape pulp (Saunier et al., 1989) and a red wine (Brillouet et al., 1990 a). Wine A was 2x more concentrated in this fraction than wine B.

Conversely, fractions II' and III' exhibited notable differences, especially at the arabinose level. This was mainly at the terminal nonreducing position in both fractions from wine B, while in wine A it was distributed between terminal, 5- and

3,5-linked positions. This latter distribution is rather typical of arabinan-like structures (Saulnier et al., 1988b). Differences were slighter at the galactose level, except for 3,4-linked galactose which was more concentrated in fractions II' and III' from wine A.

The relative rhamnose content increased sharply in fractions II' and III' from both wines, which correlated with galacturonic acid content of these fractions (Fig. 4a and 5a). Rhamnose is known to be inserted in rhamnogalacturonan acidic chains (Saulnier et al., 1988b). Relative proportions of rhamnose methyl ethers were also different, 2,3,4-linked rhamnose being detected only in II' and III' from wine B. Thus, the structure of grape neutral polysaccharides in fraction II seemed to be independent of the wine making procedure. Different patterns of degradation of rhamnogalacturonans by pectolytic enzymes in both wine making processes were evidenced in low molecular weight fractions II' and III'. Filtration of neither wines altered proportions of methyl ethers within each sugar family or for each fraction (I→III were not available from wine B due to complete retention). However relative proportions of sugars were modified after microfiltration. II and III from wine A and II' and III' from wine B were higher in mannose compared with the crude wines. This preferential retention of grape polysaccharides versus yeast mannans has been reported after microfiltration of a red wine on a membrane coated with zirconium oxide (Brillouet et al., 1989) and was not explained till now.

CONCLUSION

ALTHOUGH QUANTITATIVE and qualitative differences in polysaccharides in wines A and B did not allow a clear interpretation of their filtration profiles, hypotheses can be proposed as follows: in wine A, polysaccharides of high molecular weight (fraction II) being numerous, accumulated at the surface of the membrane and established a protecting gel (concentration layer) acting as a superimposed filtering medium which prevented fouling of the underlying alumina membrane by smaller molecules. With wine B, either external fouling was not efficient enough to prevent internal fouling due to the relative loss in high molecular weight polysaccharides, or structurally different molecules from fractions II' and III' interacted more strongly with the membrane thus limiting the filtration flux. Studies of molecules deposited at the surface and inside the membrane are needed to enlighten these hypotheses.

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Microbiological and Enological Parameters during Fermentation of Musts from Poor and Normal Grape-Harvests in the Region of Alicante (Spain)

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ABSTRACT

Must and wine from grapes harvested in two vintages (1986 and 1987) were anlyzed during vinification for physicochemical and microbiological characteristics. The 1986 vintage would be considered abnormal or poor vintage because of higher rainfall at harvest, and the 1987 one a normal vintage. Low reducing sugars and high volatile acidity at the beginning of the poor fermentation was observed as compared to normal vinification. The yeast population showed atypical evolution through the process since oxidative yeasts were isolated in the first stages of the poor vinification.

INTRODUCTION

THE TRANSFORMATION of grape juice into wine is a complex microbial reaction traditionally involving sequential development of several species of yeasts and lactic acid bacteria (LAB). The microbiological process of must fermentation has been the subject of numerous studies (Fleet et al., 1984; Lafon-Lafourcade, 1983; Poulard, 1984). However the results obtained present great variations, which could be attributed to differences in ecosystems studied, as well as different criteria employed for identification of strains (Amerine et al., 1982; Lafon-Lafourcade, 1983).

Understanding the vinification process from an enological perspective includes not only relationships among different microorganisms (yeasts, filamentous fungi, lactic acid and acetic acid), but physico-chemical parameters as well. The area we studied is located in the Alicante province, where the normal climate is characterized as warm continental with a mediterranean influence. The wines obtained are included in "Denominación de Origen" (D.O.) Alicante according to Spanish regulations. They reflect the characteristics of the grape variety Monastrell, (the 2nd most important red grape in quantity produced per area cultivated in Spain). The red wines are characterized by high concentration of ethanol and rich color. During the final stages of grape maturation in this region, dry and hot climatological conditions (maximum temperatures around 40°C) are normal. Nevertheless, heavy rains may ocassionally fall during harvest-time (September-October) causing rapid decay of grapes. All vintages carried out in the mentioned region are traditional fermentation processes, without inoculation, therefore they depend to a great extent on activity and growth of indigenous yeasts. Adverse climatic conditions at time of harvest undoubtedly affect composition of the yeast population. Very few reports are available on studies dealing with such abnormal conditions in our zone. They are concerned only with partial aspects of the elaboration procedure (Sanchez-Infante et al., 1985; Mulet et al., 1985).

Numerous studies on the microbial role in vinification have been performed to date, but little is known about relationships between microbial populations during vinification and occurence of adverse climatic conditions at final stages of grape maturation (Salgues et al., 1984). Adverse climatic conditions are responsible for deficient vinifications with serious problems arising during fermentation, usually causing incomplete fermentation.

The major aim of our study was to analyze the influence of high rainfall at time of harvest on the vinification process compared with a normal vintage. We compared enumeration, isolation and identification of yeasts, LAB and filamentous fungi from abnormal (poor) and normal must samples, at close time intervals during vinification. The main physicochemical enological parameters and their relationships to microbial content during fermentation were also studied.

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MATERIALS & METHODS

THE RED WINES studied were produced under traditional fermentation conditions along two vintages (1986 and 1987), from Monastrell variety grapes cultivated in the Alicante area.

Ten samples from red wines in 1986 and eight in 1987 were anlyzed during the winemaking procedure. Sample volumes of one litre must or wine were aseptically taken, transferred to a sterile glass container, and transported to the laboratory under refrigeration (0-4°C). Samples were analyzed within 6 hours.

In the laboratory, the following physico-chemical and microbiological parameters were analyzed for each sample:

Meteorological parameters

The temperature and rainfall values in the Alicante area during 1986 and 1987 were obtained from the "Servicio Meteorológico de la Comunidad Valenciana".

Enological parameters

The following parameters were measured according to the methodology of Ribéreau-Gayon et al. (1982): density 20°C; temperature; pH; total acidity and volatile acidity; alcohol content at 20°C (vol %); reducing sugars; free SO_2; combined SO_2; total SO_2, dry weight and content of malic and lactic acids.

Enumeration, isolation and identification of lactic acid bacteria

Lactic acid bacteria (LAB) were enumerated by the MPN (Most probable number) procedure in MRS liquid medium (Davis et al., 1986; Pardo et al., 1989), to which cycloheximide (0.05% w/v) was added to inhibit growth of contaminant yeasts and molds. The tubes were incubated at 32°C for 5 to 12 days. Isolation of LAB was carried out by surface spreading samples (0.1 ml) from the MPN tubes on to MRS agar medium. Plates were incubated at 32°C for 5 to 12 days in anaerobic jars with GasPaks. Colonies were provisionally considered LAB on the basis of morphology, gram-positive and catalase-negative reactions. A representative number of colonies was isolated and further identified. Criteria employed included cell morphology, growth characteristics, homo and heterofermentation of glucose, and ammonia production (Kandler and Weiss, 1986; Schleifer, 1986; Westhizen et al., 1981).

Identification at species level was accomplished using the API 50 CH carbohydrate galleries for lactobacilli as described by Lafon-Lafourcade and Joyeux (1979).

Table 1—Variations in temperature and rainfall in 1986 and 1987 during harvest-time

	Temperature (°C)						Rain (mm/m²)			
	1986			1987			1986		1987	
Month	Maxim	Minimal	Mean	Maxim	Minimal	Mean	Total	Maximun[b]	Total	Maximun[b]
January	19.0	−2.8	8.0	20.4	−4.0	7.6	5.6	4.7	18.0	5.5
February	21.0	−2.8	8.2	22.2	−2.8	8.9	5.0	3.2	33.7	7.8
March	25.0	0.0	10.0	27.0	−1.0	11.7	4.0	2.0	0.9	0.9
April	20.8	−1.0	11.0	26.4	13.0	18.0	20.5	12.5	5.0	5.0
May	29.0	5.2	17.8	27.0	19.4	21.0	7.0	4.0	29.0	21.0
June	33.8	13.0	22.7	33.0	10.2	20.8	17.2	7.0	ND[a]	ND[a]
July	39.8	13.0	22.7	32.6	14.0	23.2	81.2	49.2	6.3	4.0
August	37.0	15.0	24.4	37.8	13.8	24.9	0.5	0.5	3.3	1.5
September	30.8	11.6	21.6	34.4	13.4	23.1	83.2	45.0	31.0	25.0
October	25.8	5.2	15.8	26.6	5.8	16.7	93.7	26.0	32.2	18.5
November	20.6	2.8	16.3	22.2	−1.8	11.0	17.9	14.6	128.1	67.1
December	17.4	−1.8	12.0	21.0	−2.0	9.6	3.1	1.5	28.5	8.5

[a] Not determined

[b] Maximum values for rainfall refer to the total amount fallen during the most rainy day of the month.

Table 2—Changes in physicochemical parameters during fermentation of red wines (1986)

Time from start of fermentation (days)	Temp	Density 20°C	Volatile acid acetic (g/L)	Total acid (tartaric g/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	pH	Reducing sugar (g/L)	Alcoholic conc (vol %)	Malic acid conc (g/L)	Lactic acid conc (g/L)
1	23.8	1.0618	0.50	4.74	10	155	3.83	136.2	3.97	2.5	0.5
3	38.5	1.0091	0.42	5.73	15	137	3.82	27.0	10.26	2.0	1.0
5	33.2	1.0024	0.43	5.90	14	129	3.83	6.9	11.41	2.5	1.0
11	26.0	1.0002	0.42	5.96	15	125	3.87	4.2	11.59	2.5	1.0
16	20.0	0.9989	0.45	6.12	28	134	3.80	2.7	11.70	2.5	1.0
21	20.0	0.9989	0.47	6.42	25	121	3.78	2.7	11.82	1.5	2.0
34	20.0	0.9989	0.47	5.70	22	124	3.72	2.7	11.92	1.5	2.0
50	20.0	0.9989	0.51	6.27	25	128	3.83	2.8	12.09	1.0	2.0
81	20.0	0.9989	0.62	4.74	6	120	3.89	2.7	12.09	0.0	2.5
140	20.0	0.9986	–	4.33	6	120	3.89	1.9	12.10	0.0	3.0

Table 3—Changes in physicochemical parameters during fermentation of red wines (1987)

Time from start of fermentation (days)	Temp	Density 20°C	Volatile acid acetic (g/L)	Total acid (tartaric g/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	pH	Reducing sugar (g/L)	Alcoholic conc (vol %)	Malic acid conc (g/L)	Lactic acid conc (g/L)
1	21	1.0857	0.21	4.59	6	16	3.38	199.0	0.00	3.0	0.0
5	25	1.0742	0.29	5.35	6	80	3.12	170.0	0.00	2.5	1.0
7	35	1.0056	0.31	7.34	16	80	3.26	5.0	12.48	2.5	1.0
9	35	0.9994	0.35	7.30	16	80	3.28	2.5	13.21	2.5	1.0
21	28	0.9950	0.35	7.12	10	75	3.30	2.0	13.50	2.5	2.0
26	24	0.9934	0.41	6.12	11	60	3.32	1.5	14.27	1.5	2.0
141	23	0.9917	0.47	5.05	13	89[a]	3.30	1.5	14.38	1.0	2.5
209	20	0.9918	0.39	5.20	11	92	3.46	1.5	14.38	1.0	3.0

[a] SO_2 added to conserve wine produced.

Enumeration, isolation and identification of moulds

After dispersion and homogenization in sterile Ringer solution added with 0.01% (v/v) of Tween 80, the samples were inoculated with 0.1 mL aliquots of serial dilutions (from 10^{-1} to 10^{-6} in 1% w/v NaCl solution) onto replicate plates of Malt extract agar (2%). The plates were incubated at 28°C for 5 days and examined for fungal growth.

Fungal colonies were identified according to Raper and Fennell (1965) for *Aspergillus* and Pitt (1979) for *Penicillium*.

Enumeration, isolation and identification of yeasts

Yeasts were enumerated by spread inoculating 0.1 ml aliquots of various dilutions of the wine samples (from 10^{-1} to 10^{-6} in 1% (w/v) NaCl solution) onto plates of malt extract agar (MEA) (Kreger-van Rij, 1984). Plates were incubated at 28°C for 5 days. The various colony types were enumerated, and the selection of colonies for identification was carried out according to Snedecor and Cochram (1982). The method of Kreger-van Rij (1984) was used for identification of yeasts.

RESULTS & DISCUSSION

Meteorological parameters.

Temperature and rain data are shown in Table 1. Significant differences between rain data from both years can be observed for September and October, during harvest-time. The total rain during harvest-time of 1987 was 63.2 mm m^{-2} in contrast to 176.9 mm m^{-2}, three times as much, during harvest-time of 1986. Temperature values were normal for September and October in the Alicante region. Mediterranean climate is characterized by irregular distribution of rainfall with sudden heavy rains. The values in Table 1 give more information on type of climate.

Enological parameters

Tables 2 and 3 indicate results of the physico-chemical analyses carried out on red wines during 1986 and 1987 vinifications. High temperatures were recorded during stormy fermentation in both vinifications (38.5°C in 1986 and 35°C in 1987). During the subsequent stages, a steady decrease in temperature was recorded. Total SO_2 added at the beginning of fermentation was different in each vinification (155 mg/l in 1986 and 80 mg/L in 1987) due to spoiled grapes harvested during 1986. The SO_2 values corresponding to the vintage of 1986 decreased to a final level of 120 mg/L. In 1987 they showed a steady decrease to 60 mg/L. After fermentation, SO_2 was again added to preserve the wine. As seen in Tables 2 and 3, volatile acidity reached higher values than expected

(0.5g acetic acid/L in red wines from 1986 and 0.4 g/L from 1987); during fermentation, a slight increase was recorded. The European Economic Community (ECC) limits the volatile acidity in red wines to 0.98 % (w/v) (DOCE, 1982). In our study, the acetic acid concentration in red wines in 1986 was higher than in 1987 (0.62 g/L versus 0.39 g/L). Ribéreau-Gayon et al. (1982) considered normal values around 0.4 g/l in a vinification process. Our results show the 1986 wines exhibited considerably higher values, but the 1987 wines were still within the usual limits for volatile acidity. According to Ribéreau-Gayon et al. (1982), volatile acidity reflects the phytosanitary condition of the grapes. In our case, the high values obtained for the red wines could reflect abnormal characteristics of the grapes due to adverse climatological conditions.

Density values recorded at beginning of fermentation were lower in 1986 than in 1987, where they corresponded to normal (Mulet et al., 1985). The 1986 samples showed a marked decrease to around 0.9989, whereas the 1987 decreased less sharply, following a normal pattern (Amerine et al., 1982 and Lafon-Lafourcade, 1983). Three typical phases were distinguishable: (a) the first phase (5 to 10 days) occurred before fermentation and was characterized by low levels of degraded sugars; (b) the second phase lasted about 5 days and occurred during fermentation process, in which the sugar content decreased paralleled by an increase in alcohol; (c) The last phase occurred when fermentation stopped leaving a residue of unmetabolizable sugar (Bureau et al., 1982). In our study, as a consequence of adverse climatological conditions, some important differences were observed in the physicochemical parameters. The most striking feature was absence of the first phase. The fermentation process started from the very beginning, which could be deduced from the abnormally low sugar concentration and abnormally high alcoholic content encountered at first sampling. This finding was likely due to the effect of adverse metereological conditions on the phytosanitary characteristics of the grapes during harvest. Rupture of the grape skin causes the yeasts to contact flesh, thus to develop before the wine-making process begins.

The rest of the physicochemical parameters did not show great differences in comparison with normal fermentations.

Evolution of lactic acid bacteria during fermentation

According to Wibowo et al. (1985) there are three general procedures to isolate LAB from wines. Previous experiments in our laboratory (Pardo et al., 1989) had shown the enrichment method was more convenient than direct plating when low numbers were present. Therefore we chose the MPN procedure using MRS broth to enumerate the LAB. The evolution of LAB content in both wines during fermentation was quite similar (Fig. 1), although a peak quite at the final stages of fermentation was observed for the normal process. Except for this peak, LAB were present at low levels during the whole fermentation processes (around 10^3 CFU/mL). The high concentrations of SO_2 used for vinification could be responsible, as Davis et al. (1986) found concentrations greater than 50 mg/L restricted the growth of LAB. LAB in wines are exposed to several negative factors, such as pH, ethanol and SO_2 concentration, and temperature, as has been discussed by Davis et al. (1986). During the initial stages of fermentation, *Lactobacillus plantarum* was dominant, whereas during the final stages *Leuconostoc oenos* was most abundant. In our study, in accordance with results of others, during storage the decrease in SO_2 concentration and temperature enhanced growth of *Leuconostoc oenos*. According to Lafon-Lafourcade, levels around 10^8 LAB/ml are necessary for a malic acid fermentation. With lower numbers around 10^4 LAB/mL, this process would be slowed considerably, up to 200 days. In our study, the low numbers of LAB throughout the fermentation could be responsible for this slow decrease of malic acid.

Evolution of filamentous fungi during fermentation

The numbers of filamentous fungi were nearly as high as those of yeasts at the beginning of fermentation ($6x10^7$ in 1986 cfu/ml and $3.3x10^6$ CFU/mL in 1987). Nevertheless, they quickly showed a marked decrease after 3 to 5 days reaching undetectable levels by Plate count. The only two genera identified were *Aspergillus* and *Penicillium*. High numbers of filamentous fungi were encountered in the first samples taken. However, this number rapidly decreased as a result of high concentrations of SO_2 (Schoper and Aerny, 1985). Other factors that contributed were the anaerobic conditions and the inhibitory effect of ethanol (Amerine et al., 1982). The injured conditions of the grapes during harvest enhanced proliferation of filamentous fungi during the first phases of fermentation, but they did not affect normal development of the subsequent vinification.

Evolution of yeasts during vinification

Table 4 shows the growth cycle of yeasts in the wines during fermentation. The yeast population in the musts was initially high, $3.5x10^8$ CFU/mL in 1986, and $6x10^5$ CFU/mL in 1987. They showed a very short phase of multiplication up to $4.8x10^9$ CFU/mL in 1986 and $3.4x10^7$ CFU/mL in 1987. Instead of the classical stationary phase after maximum growth, the yeasts decreased to around 10^6 CFU/mL, followed by a short stationary phase (between 18 and 40 days). Then the decline phase reduced the yeast population to 10^2 CFU/mL in 1986 (Fig. 1). During 1987 this stationary phase was not observed. According to Ribéreau-Gayon (1985), three major phases in the growth cycle of yeasts can be distinguished during wine fermentation: (a) a limited phase of multiplication that lasts for two to five days and increases cell population to around 10^7 to 10^8 cells/mL; (b) a stationary phase, where cell count remains almost constant, which lasts about eight days; (c) a decline phase where the yeast population progressively decreases to around 10^5 cells/mL. This last phase may be prolonged up to several weeks, as can be seen in Fig. 1. In our study, in 1986, the stationary phase was very short and the decline phase very pronounced. The abnormal contitions of the grapes during harvest were undoubtedly the reason for early proliferation of microflora, and therefore for the begining of fermentation before arrival of the grapes at the cellar, as has been indicated by Salgues et al. (1984). According to Nishino et al., (1985), the high sugar concentrations, together with high temperatures and ethanol content seemed to act synergistically to inhibit yeast growth and to decrease fermentative activity. In our study,

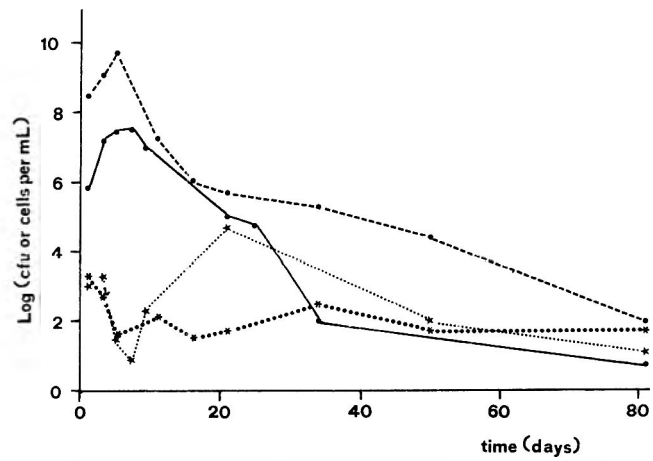

Fig. 1—Evolution of yeasts and lactic acid bacteria during fermentation of red wines (1986 and 1987). Symbols: (●----●) growth of yeasts during 1986 and (———*) during 1987; (*●●●●*) growth of lactic acid bacteria during 1986 and (*·····*) during 1987.*

Table 4—Evolution of yeasts species during fermentation in red wines

Days	Species	Frequency (%) 1986	1987
1	*Candida stellata*	0.0	6.3
	Hanseniaspora uvarum	25.0	12.3
	Kloeckera apiculata	0.0	9.4
	Kloeckera javanica	50.0	0.0
	Pickia membranaefaciens	0.0	6.3
	Rhodotorula minuta	0.0	3.2
	Saccharomyces cerevisiae	25.0	62.5
3	*Brettanomyces custersianus*	12.5	–
	Candida guillermondii	12.5	–
	Candida stellata	25.0	–
	Dekkera bruxeciensis	12.5	–
	Rhodotorula minuta	12.5	–
	Saccharomyces cerevisiae	12.5	–
	Torulospora delbrueckii	12.5	–
5	*Candida stellata*	0.0	14.3
	Saccharomyces cerevisiae	100.0	85.7
7	*Saccharomyces cerevisiae*	–	100.0
9	*Saccharomyces cerevisiae*	–	100.0
11	*Saccharomyces cerevisiae*	100.0	–
16	*Saccharomyces cerevisiae*	100.0	–
21	*Saccharomyces cerevisiae*	50.0	100.0
	Torulospora delbrueckii	50.0	0.0
26	*Saccharomyces cerevisiae*	–	100.0
34	*Saccharomyces cerevisiae*	100.0	100.0
50	*Saccharomyces cerevisiae*	100.0	–
81	*Saccharomyces cerevisiae*	100.0	–
140	*Saccharomyces cerevisiae*	100.0	100.0
209	*Saccharomyces cerevisiae*	–	100.0

this could be the reason for the small amount of residual sugars that remained undegraded up to the final samplings.

The yeast species identified in 1986 are indicated in Table 4. *Hanseniaspora uvarum, Kloeckera javanica,* and *Saccharomyces cerevisiae* were the dominant yeasts in freshly extracted must of 1987, whereas *Kloeckera javanica*, was the most abundant species (50%) from 1986. The changes in individual species during fermentation of the wine were as follows: (a) a rapid decline and disappearance of *Kloeckera javanica*; (b) an increase in yeast diversity during stormy fermentation; (c) a slow initial proliferation of *Candida stellata* followed by a gradual decline of this species; (d) the rapid growth of *Saccharomyces cerevisiae*, which was the dominant species during fermentation.

Table 4 shows the yeast species present in 1987. During the fermentaion phase the most abundant species were *Saccharomyces cerevisiae* (62.5 %), *Hanseniospora uvarum* (12.3%) and *Kloeckera apiculata* (9.4%). The most important feature was the dominant role of *Saccharomyces cerevisiae* during fermentation. It represented 85.7% of the total yeast population cells during the stormy fermentation phase and reached 100% at the end of fermentation. The numbers of yeasts in grapes and their evolution are influenced by several factors. Among them, the latitude of the vineyard and the climatological characteristics play an important role. In hot years *Kloeckera* spp. predominates, whereas in cold years *Hanseniaspora* spp. is dominant (Poulard et al., 1981). We found *Kloeckera* to be predominant in the area studied, where a hot summer together with the continental climatic conditions, had an important influence.

As a consequenc of the injured conditions of the grapes, the number of oxidative yeasts increased as previously reported by Poulard et al. (1980). In 1987 we found less diversity of oxidative yeasts, because of less altered conditions of the grapes as has previously been mentioned. *Saccharomyces cerevisiae* was isolated from the very beginning of fermentation, which may be due to high temperatures as well as high concentrations of SO_2 used in the vinification. In fact, Sanchez-Infante et al. (1985) found more sporulating strains of yeast in regions with warm climatic conditions than in colder areas. On the other hand, high concentrations of SO_2 have been selective for *Saccharomyces cerevisiae* as other species do not grow in these conditions (Poulard et al., 1980). This dominance of *Saccharomyces cerevisiae* from the very early stages was probably responsible for the rapid fermentation of the must (Sanchez-Infante et al., 1985).

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Identification and Reduction of Ellagic Acid in Muscadine Grape Juice

T.Y. LIN and R.P. VINE

ABSTRACT

The precipitate which forms during storage of clarified and pasteurized Magnolia and Carlos muscadine juices was examined using paper chromatography, HPLC, and ultraviolet, infrared, and mass spectrometries. The methanol washed juice precipitate was identified as ellagic acid. The concentrations of ellagic acid in Magnolia juice treated with polyvinylpolypyrrolidone (PVPP) at 0.12, 0.36, 0.60, 0.84, and 1.08 g/L and gelatin at 0.06, 0.12 and 0.24 g/L were determined. The treatment using 1.08g PVPP/L was most effective in reducing ellagic acid, total phenolics, and juice browning.

INTRODUCTION

ELLAGIC ACID, an acid hydrolytic product of ellagitannin (Singleton and Marsh, 1966), has been studied extensively in the wine industry as a precipitate caused by wood treatment or during traditional wine aging in oak barrels (Pocock et al., 1984). It has been reported in many fruits including strawberries, Brazil nuts and raspberries (Hennig and Burkhardt, 1957). However, no report has been released concerning presence of ellagic acid in grape varieties.

A preliminary study revealed a precipitate in clarified, filtered, and pasteurized muscadine grape juice (Harkness, 1989). In that study, the precipitate increased in processed muscadine juice and caused notable sediment after 3 months at 13°C. This precipitation problem has also been obsserved in many muscadine wines from wineries of the southeastern United States (Harkness, 1989).

Various fining agents have been used to clarify beverages, including gelatin, isinglass, and certain synthetic resins such as polyamide, and polyvinylpolypyrrolidone (PVPP), which have selective affinity for tannins. Proanthocyanidins, and other polyphenols function through hydrogen bonding and other linkages (Mennett and Nakayama, 1969; Lindsay, 1985). De Villiers (1961) and Harris and Ricketts (1959) studied finely divided Nylon as a fining agent for white wines and beers, and reported Nylon could remove leucoanthocyanins and other polyphenolic compounds from those beverages. Caputi and Peterson (1965) found a significant decrease in degree of browning on PVP treated Palomino and French Colombard wines. However Hoey and Codrington (1985) reported no reduction of hydrolyzable tannins in white wines treated with bentonite, polyclar, isinglass, or silica hydrogel.

The objective of our study was to identify the precipitate in muscadine juices using paper chromatography, HPLC, and ultraviolet, infrared, and mass spectrometries. The effect of PVPP and gelatin in reducing the juice precipitate was also studied, as an initial attempt to eliminate the problem.

MATERIALS & METHODS

Juice production

Seven hundred kilograms of mature muscadine grapes (*Vitis rotundifolia* Michx., var. "Magnolia" (13.2° Brix, pH 3.3, and 0.54 g/100 mL titratable acidity) and "Carlos" (13.4° Brix, pH 3.4, and 0.42 g/100 mL titratable acidity)) were harvested from research farms at the Mississippi Agricultural & Forestry Experiment Station, Mississippi State Univ., in September 1987. After storage at 13°C overnight, the grapes were crushed and mixed with potassium metabisulfite to make a final concentration of 50 ppm free sulfur dioxide in the juice as determined by the Ripper method (Zoecklein et al., 1980). The grapes were than pressed by a vertical basket press at 60 atm and stored at −3°C in 18.9 liter carboys. After settling overnight, the juices were racked into 1.9 liter jars and stored at −9°C 1 month before use.

Seventeen liters of each juice were thawed, treated with bentonite (0.12g /L juice), stored overnight at −3°C, racked, filtered through a glass fiber filter and a 0.45 μm membrane filter and bottled in 220 mL juice bottles. Since our study focused on precipitate formed after juice was bottled, all juices were prefiltered before bottling. Those juices were pasteurized at 85°C for 10 min in a Consolidated Model SSR-5A Sterilizer and stored at −3°C 6 months. The temperature of −3°C for juice storage was not a requirement for formation of the precipitate.

Identification of the precipitates

Processed Magnolia and Carlos juices were filtered separately through a 0.45 μm membrane filter and the separated precipitate was suspended in water. The suspension was then re-filtered through a 0.45 μm membrane filter and using vacuum filtration washed by water and methanol to remove impurities. Due to its low solubility in water and alcohol (Windholz, 1983), ellagic acid was presumed to be the only compound left on the membrane filter after juice precipitate was washed by water and methanol. The sediments due to tannin - protein interaction as reported in many natural beverages (White, 1957; Bate-Smith, 1973) should be removed after washing. The color of the precipitate on the filter membrane was brown after washing. The precipitate was dissolved in methanol by mixing on a Corning stirrer 24 hr.

Paper Chromatographic Analysis. The juice precipitate in methanol and standard ellagic acid were spotted on Whatman No. 3 chromatographic papers. The developing solvents were n-butanol: 27% acetic acid (1:1, v/v) (BAW) in the first direction and 6% acetic acid (6HA) in the second direction. The chromatograms were viewed under short-wave UV light (254 nm). Chromogenic sprays were a "base mixture" of saturated $NaHCO_3$:20% Na_2CO_3 (2:1, pH 9.6); "NSSC" of 15g Na_2SO_3 and 3.5g Na_2CO_3 in 350 mL water; pNA (0.05% p-nitrobenzendiazonium tetrafluoborate in water); $FeCl_3$ (1%)-$K_3Fe(CN)_6$ (0.3%) (1:3); Gibbs' reagent (0.1% methanolic 2,6-dichloroquinone-4-chloroimide) oversprayed with lead subacetate solution diluted with methanol (1:5, v/v) (Hart and Hillis, 1972); a freshly prepared saturated KIO_3; and $SbCl_3$ (2% in $CHCl_3$) (Seikel and Hillis, 1970). The chromatograms, after being sprayed by aq. KIO_3 and $SbCl_3$, were viewed under long-wave UV light (366 nm).

HPLC Analysis. Retention time of the precipitate was determined according to a modification of the HPLC method published by Salagoity-Auguste (1986). Two linear gradient systems were used: (1) 10% methanol to 98% methanol for 15 min, and (2) 10% acetonitrile to 98% acetonitrile for 15 min. The flow was 0.8 mL/min. The injection volume was 10 μL, and the wavelength of the detector was 255 nm. Instrumentation for analyses was A Varian Vista 5500 HPLC system (Walnut Creek, CA) equipped with a Waters uBondapak phenyl column (Milford, MA) (Coign, 1988), Rheodyne automatic loop injector (Cotati, CA), Varian 8085 autosampler, UV-200 inboard variable wavelength scanning detector, instrument interface module - A, and a DS 604 data system. A Hewlett-Packard ThinkJet printer was used to print out the chromatogram.

Ultraviolet spectrometric analysis. The precipitate in methanol and the ellagic acid standard were filtered through 0.45 μm membrane

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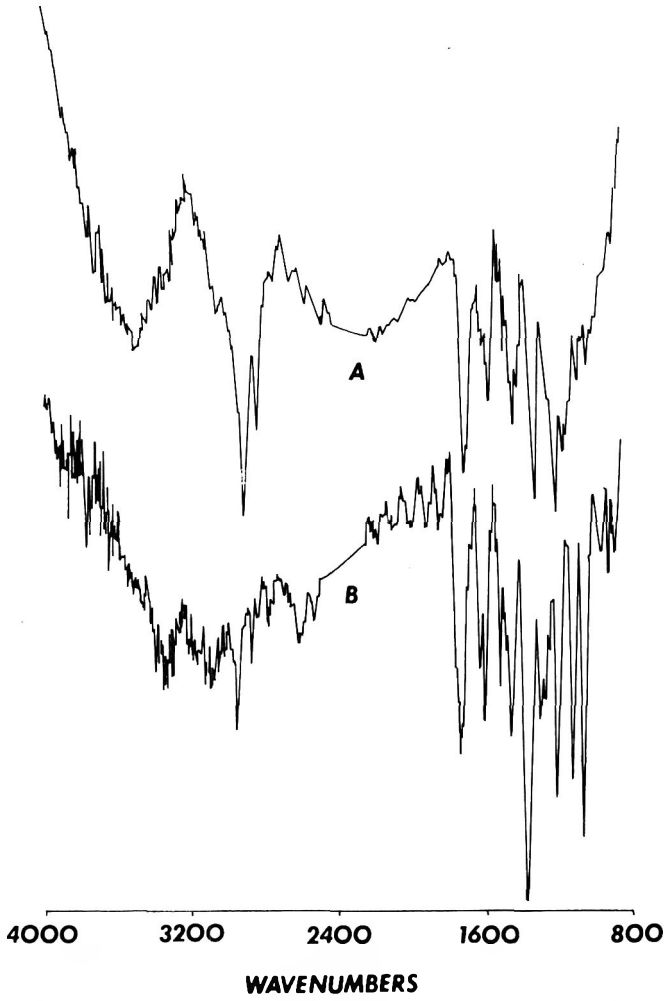

Fig. 1—Infrared spectra of (A) Magnolia juice precipitate and (B) ellagic acid standard.

filters. Samples were then scanned from 500 nm to 210 nm by a Bausch & Lomb spectronic 2000 spectrophotometer and the wavelengths of the maximum absorption were determined.

Infrared spectrometric analysis. The infrared spectra of the juice precipitate and the ellagic acid standard were obtained using a Nicolet 7199 Fourier Transform Infrared Spectrometer (FT-IR) with a Nicolet 1280 computer. A cast film technique was applied and a KBr pellet was used as the cell to support the sample. Methanol was used to dissolve the sample. Range of scan was 4000 cm^{-1} to 800 cm^{-1}. Resolution was set at 4 cm^{-1} and the number of scans for each analysis was 32. Liquid nitrogen was used to cool the MCT detector.

Mass spectrometric analysis. Mass spectra were reported on a Finnigan 4500 quadrupole mass spectrometer equipped with an electron impact source. A solid probe technique was used to introduce the sample. The temperature of the probe tip was set at 440°C. The instrument parameters were: electron energy 70 V, source pressure 300 mTorr, electron multiplier 1100 V, and an ionization chamber at 250°C.

PVPP and gelatin treated juices

PVPP (Presque Isle Wine Cellars, North East, PA) and gelatin (75 bloom) (Scott Lab., San Rafael, CA) were mixed with raw Magnolia muscadine juice at 0.12, 0.36, 0.60, 0.84, and 1.08g PVPP/L juice, and 0.06, 0.12, and 0.24g gelatin/L juice for 30 min at speed 5 on a Corning stirrer at 25°C. The gelatin suspension was prepared by suspending 0.5% gelatin in deionized water preheated at 48°C. No preparation was needed for PVPP. The juice samples were then filtered through a 0.45 μm membrane filter and bottled in 220 mL juice bottles. These juices were pasteurized at 85°C 10 min in a sterilizer and stored at −3°C 7 days.

The concentration of ellagic acid in juice was determined by the HPLC method. Ellagic acid was extracted from a 20 mL juice sample by ethyl acetate (Salagoity-Auguste et al., 1986), and the concentration was analyzed by HPLC (1) described previously. The measurement was performed in triplicate. The method of Singleton and Rossi (1965) was employed for determination of total phenols. Folin-Ciocalteu reagent was used and a Bausch & Lomb Spectronic 21 spectrophotometer was set at 765 nm for absorbance measurement. Degree of browning was measured by the spectrophotometer after juice was filtered through a 0.45 μm membrane filter (Amer ne and Ough, 1980) and was expressed by absorbance at 420 nm.

Statistical analysis

All data were subjected to analysis of variance for one-factor, completely randomized design and Duncan Multiple Range Test using SAS (Joyner, 1985).

RESULTS & DISCUSSION

Identification of ellagic acid

The precipitate in the juices consisted of tiny granulated and filament-like particles, white to slightly yellowish. No brown sediment was seen at the end of 6 months storage. Both juice precipitate and the ellagic acid standard had the same Rf values of 0.46 in BAW and 0.02 in 6HA. Colors of the developed spot under short-wave UV (254 nm) were faint violet and after being sprayed by a base mixture light yellowish green, similar to Seikel and Hillis' findings (1970). With pNA and saturated KIO_3, the weak ellagic acid spot could be detected easily and colors were light yellow and light pink, respectively. An intense pink was observed under long-wave UV after being sprayed by KIO_3. This spot showed yellowish in color after spraying with Gibbs' reagent. None of the spots oversprayed by $SbCl_3$ and $FeCl_3$-$K_3Fe(CN)_6$ were visible. However, a light blue color was evident when the developed spot was exposed to long-wave UV after spraying with $SbCl_3$.

In HPLC analysis both the juice precipitate and ellagic acid standard showed peaks at the same retention time, 14.7 min using methanol and pH 2.5 water as eluting solvents, and 7.8 min when of acetonitrile and pH 2.5 water were used as eluting solvents.

Spectrometric Analysis

In ultraviolet analysis two peaks at the same wavelengths of 255 nm and 355 nm were found on the UV spectra of both juice precipitate and the ellagic acid standard. In infrared analysis all major peaks on the spectra of both sample and the ellagic acid standard were found at the same wavenumbers (3321, 2915, 2840, 1719, 1590, 1503, 1441, 1355, 1281, 1102-1047, and 911 cm^{-1}) (Fig. 1). The peak at 3500 cm^{-1} on the spectrum of the sample was due to presence of water in the precipitate. The relative intensities of those peaks on the spectra of the precipitate and the ellagic acid standard were similar except the peak at 1281 cm^{-1}, which probably was due to presence of impurities such as proteins in the juice precipitate.

In mass spectrometric analysis as shown in Fig. 2, a large peak at 70 scan time was found on the total ion current (TIC) chromatogram of the precipitate and the temperature was 440°C at 70 scan time, identical with the 440°C found at peak of 86 scan time on the TIC chromatogram and the heating curve of the ellagic acid standard. This indicated an extremely high temperature was required to evaporate this precipitate into the ionization chamber and this vaporizing temperature was the same as that of the ellagic acid standard. The molecular ion peak was found at m/z of 302 on the mass spectra of both juice precipitate and the standard, which corresponded to the molecular weight of ellagic acid. All peaks on both mass spectra also matched.

Effect of PVPP and gelatin in reducing ellagic acid

The addition of 1.08g PVPP/L juice was most effective among all treatments for lowering ellagic acid content (Table 1). No

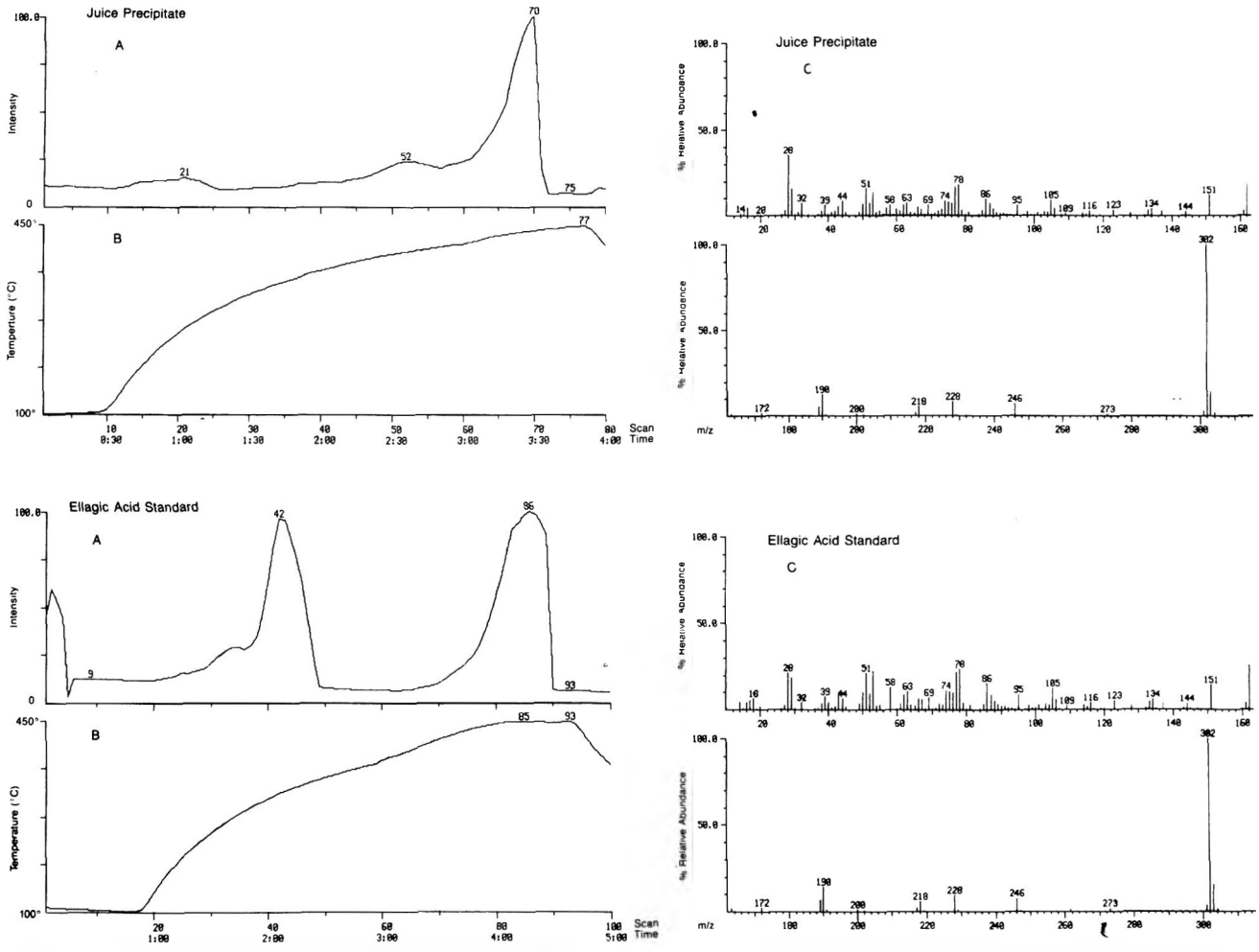

Fig. 2—(A) Total ion current, (B) ballistic heating curve, and (C) mass spectrum of Magnolia juice precipitate and ellagic acid standard.

Table 1—Ellagic acid, total phenols, and degree of browning (absorbance at 420 nm) in processed muscadine grape juice treated with PVPP and gelatin

Treatments	Ellagic acid (ppm)	Total phenols (ppm)	Absorbance (420 nm)
PVPP (g/L)			
0	20.6[a]	518.3[a]	0.12[a]
0.12	17.0[bc]	475.4[b]	0.09[b]
0.36	17.0[bc]	438.7[c]	0.08[bc]
0.60	17.6[bc]	381.2[d]	0.07[cd]
0.84	15.2[c]	349.8[e]	0.06[d]
1.08	12.0[d]	340.6[e]	0.06[d]
Gelatin (g/L)			
0	20.6[a]	518.3[a]	0.12[a]
0.06	18.2[ab]	508.4[a]	0.09[b]
0.12	16.0[bc]	473.4[b]	0.09[b]
0.24	16.7[bc]	475.6[b]	0.10[ab]

[a–e] Values within columns with similar superscripts are not significantly different ($p<0.05$) by Duncan Multiple Range Test.

significant difference ($p>0.05$) on removing ellagic acid was found among the treatments with PVPP or gelatin. However, all PVPP treatments reduced ellagic acid significantly ($p<0.05$) compared with non-treated juice. Similar results were found in gelatin clarified juice, except that ellagic acid content in juice with 0.06g gelatin was not significantly different ($p>0.05$) from non-treated juice.

The removal of total phenolic compounds was directly proportional to the level of PVPP used in the juice treatments and the addition of 0.84 and 1.08g PVPP/L juice showed significantly ($p<0.05$) more removal of total phenolic compounds than other PVPP treatments. Juices treated with 0.12 and 0.24g gelatin/L were found only slightly lower in total phenolic compounds than those treated with 0.06 g gelatin or not treated. Treatments involving addition of 0.36 or more g PVPP/L were significantly more effective ($p<0.05$) than any of the gelatin treatments.

The degree of browning was reduced significantly ($p<0.05$) in muscadine juice treated with either PVPP or gelatin. The concentrations of total phenolic compounds significantly correlated (coefficient = 0.957 < 0.0001) with the degree of browning. The lower the concentrations of total phenolic compounds in muscadine juice, the lower the degree of browning, which corresponded with findings of Amerine et al. (1980).

Though the treatment using 1.08 g PVPP/L juice was the best in reducing ellagic acid, this only lowered the ellagic acid and did not totally remove it. The problem has not yet been eliminated. Further research is needed to determine effects of PVPP and other fining agents, heat, and pressure in reducing/producing ellagic acid precipitate in both processed and unprocessed muscadine juices. Investigation of ellagic acid precursors in the muscadine juice is also needed.

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Effects of Variety, Maturity and Processing on Pear Juice Quality and Protein Stability

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ABSTRACT

d'Anjou, Comice, and Bartlett pears were processed into clarified juices from hard green and soft ripened fruits. Processing trials with and without SO_2 were conducted. Effects of variety, maturity and processing on browning, turbidity, proteins, and stability were investigated. Browning increased with heat treatment, and was reduced by processing with SO_2. Total soluble protein content increased with fruit maturity and processing with SO_2. Protein fractions from a clarifying enzyme preparation (mainly arabinase and amylase) with MW in the range of 64,000—92,000 and containing glycoproteins were not removed by fining/clarification. They contributed to protein instability in the clarified pear juices, but could be removed by heat treatment (pasteurization) prior to final filtration and bottling. Our results indicate some enzymes added for clarification during processing may contribute to post-clarification haze and sediment formation.

INTRODUCTION

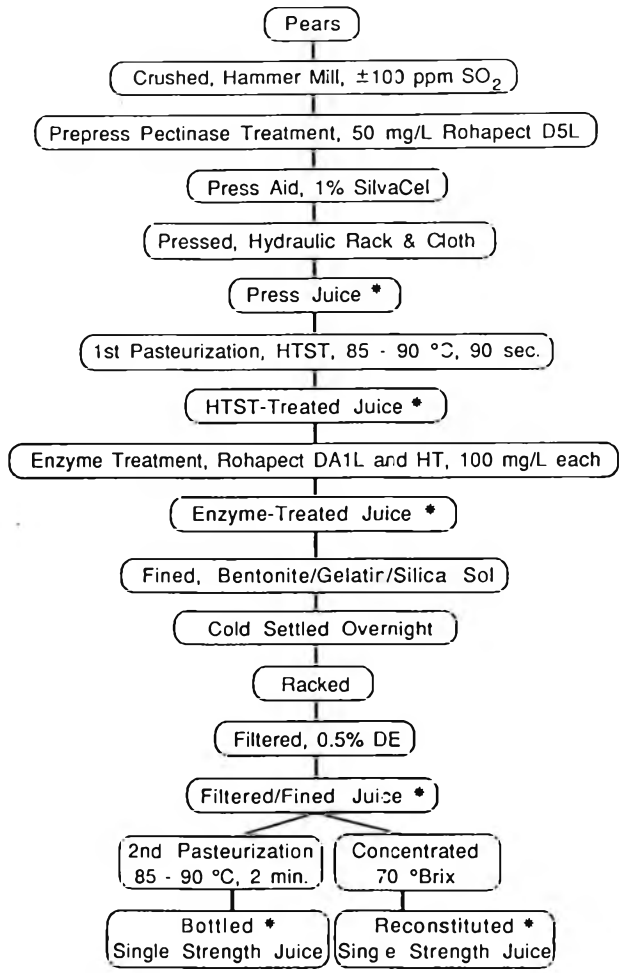
PEARS are one of the major fruits produced in the western USA. During processing and storage of pear juices, browning and instability (turbidity) are major concerns which limit commercial utilization and reduce economic value. Browning developing during processing is due to the action of polyphenol oxidase (Tate et al., 1964; Rivas and Whitaker, 1973; Halim and Montgomery, 1978), while color deterioration during storage is due to the Maillard reaction (Cornwell and Wrolstad, 1981).

Of additional concern are soluble proteins which become less soluble and form haze/sediment which reduces quality and acceptability of juices. Hsu and Heatherbell (1987) and Hsu et al. (1989) indicated the soluble proteins in grape and apple juices were important to juice stability. However, there is a lack of information concerning the nature of proteins in pear juices. The purposes of our study were to investigate the effects of variety, maturity and processing on browning, tubidity, protein and juice stability.

MATERIALS & METHODS

Preparation of juices

Bartlett, Comice, and d'Anjou pears obtained from the Mid-Columbia Experiment Station, Hood River, Oregon, were stored at 1±2°C before processing. For ripening, fruits were removed from cold storage and held in a ripening room (18°C, 90% humidity) for 1 wk. Hard green and ripened fruits were processed into juices as described by Hsu et al. (1989) in the pilot plant of Dept. of Food Science at Oregon State University. All enzymes for processing were obtained from Rohm Tech, Inc., Malden, MA. Duplicate 30–35 kg lots were crushed for each trial. Comice and d'Anjou were processed from hard green fruits with and without addition of 100 mg/L SO_2 into juices by spraying a 1.8% solution of potassium metabisulfite onto the fruit at crushing. One percent (v/v) solution of Rohapect D5L pectinase was added to a final concentration of 50 mg/L and reacted at room temperature (20–23°C) 90 min before pressing. Thirty minutes before pressing, 1% (w/v) SilvaCel press aid was added to the mash. Pressing was carried out on a hydraulic rack and cloth press. Particles were removed from juice by racking through a 100 micron mesh bag. Juices were heated in an APV HTST unit to 85–90°C for 90 sec and cooled to 40°C. Enzyme clarification consisted of treatment with 100 mg/L each of Rohapect DA1L (a pectinase arabinase mixture) and Rohapect HT amylase. Juices were maintained at 45°C until gel-forming pectin was no longer detected by alcohol test (Anonymous, 1982). All juices were fined (clarified) with bentonite at 500 mg/L (sodium bentonite, Volclay), gelatin at 100 mg/L (Scott Labs, San Rafal, CA), and silica sol at 300 mg /L (clarifying agent C-2, Rohm Tech. Inc.). About 20 min reaction time was allowed between fining treatments. Juices were racked after settling overnight at 5°C and filtered with 0.5% SuperCel DE (Manville, Denver, CO) and divided into two parts. First part of filtered juices was bottled and heated at 85–90°C for 2 min as bottled juices. Second part of filtered juices was concentrated to 70 °Brix with a rotory evaporator. The flow diagram for processing is shown in Fig. 1. Juice samples were obtained from each processing stage and ana-



* Indicates the stage at which juice samples were collected for analysis.

Fig. 1.—Flow diagram of pear juice pressing.

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Table 1—Effect of variety, maturity, and processing on Hunter turbidity reading[a] and visible turbidity[b] of pear juices

Juice samples[c]	Press juice	HTST-treated	Enzyme-treated	Fined/filtered	Bottled	Reconstituted
d'Anjou						
Hard green ($-SO_2$)	76.5(+ + +)	88.7(+ + + +)	90.1(+ + + +)	0.1(−)	0.7(+ −)	18.3(+ +)
Hard green ($+SO_2$)	97.5(+ + + +)	99.2(+ + + +)	99.2(+ + + +)	0 (−)	0.6(+ −)	26.9(+ +)
Soft ripen ($-SO_2$)	86.7(+ + + +)	85.2(+ + + +)	90.0(+ + + +)	0 (−)	0.8(+ −)	76.8(+ + +)
Comice						
Hard green ($-SO_2$)	84.1(+ + + +)	92.9(+ + + +)	94.5(+ + + +)	0 (−)	0.9(+ −)	15.0(+ +)
Hard green ($+SO_2$)	97.7(+ + + +)	98.7(+ + + +)	99.3(+ + + +)	0.4(−)	1.4(+ −)	21.1(+ +)
Soft ripen ($-SO_2$)	87.3(+ + + +)	88.8(+ + + +)	92.4(+ + + +)	0 (−)	2.0(+ −)	66.5(+ + +)
Bartlett						
Hard green ($-SO_2$)	99.9(+ + + +)	99.0(+ + + +)	99.4(+ + + +)	0.1(−)	2.2(+ −)	46.0(+ + +)
Soft ripen ($-SO_2$)	98.6(+ + + +)	99.2(+ + + +)	99.3(+ + + +)	0.1(−)	2.7(+ −)	83.0(+ + + +)

[a] Values are means of duplicate processing trials.
[b] Turbidity of visible observation indicated in brackets ( ): (−) = Clear; (+ −) = Trace Haze (only detected under strong light); (+) = Slight Haze; (+ +) = Moderate Haze; (+ + +) = Strong Haze; (+ + + +) = Extreme Haze.
[c] Refer to Fig. 1

Table 2—Effect of variety, maturity and processing on browning[a] of pear juices as measured by absorbance at 420 nm

Juice samples[b]	Fined/filtered	Bottled	Reconstituted
d'Anjou			
Hard green ($-SO_2$)	0.134 ± 0.004	0.151 ± 0.010	0.293 ± 0.001
Hard green ($+SO_2$)	0.036 ± 0.002	0.077 ± 0.003	0.138 ± 0.004
Soft ripen ($-SO_2$)	0.127 ± 0.002	0.149 ± 0.015	0.339 ± 0.002
Comice			
Hard green ($-SO_2$)	0.174 ± 0.002	0.187 ± 0.002	0.275 ± 0.015
Hard green ($+SO_2$)	0.068 ± 0.002	0.089 ± 0.001	0.136 ± 0.008
Soft ripen ($-SO_2$)	0.196 ± 0.003	0.206 ± 0.001	0.322 ± 0.003
Bartlett			
Hard green ($-SO_2$)	0.372 ± 0.008	0.397 ± 0.003	0.508 ± 0.007
Soft ripen ($-SO_2$)	0.354 ± 0.006	0.410 ± 0.011	0.525 ± 0.052

[a] Values are means + SEM (standard error of mean) for n = 2 processing trials. H_2O was used as blank.
[b] Refer to Fig. 1.

lyzed immediately. Concentrates were reconstituted to single strength juices with the same degrees of Brix as bottled juices before analysis.

Determination of turbidity

Turbidity was measured by visual observation under a strong beam of light and by a Hunter Model D25P-2 Color Difference Meter in the transmission mode (Hsu and Heatherbell, 1987b). Sediments in juice samples were resuspended before measurement.

Determination of browning

The degree of browning was determined from absorbance at 420 nm by using a Perkin-Elmer 550 spectrophotometer. Absorbance at 700 nm was subtracted (Wrolstad, 1976) to correct for turbidity.

Determination of total soluble proteins

All juice samples were filtered through 0.45 μm membrane before analysis. Total soluble proteins were determined using a modified Bio-Rad procedure described by Hsu and Heatherbell (1987a).

Heat stability test

The heat stability of juices was determined by the procedure recommended by Pocock and Rankine (1973). Fifty mL of filtered juices were transferred into 65 mL bottles. Bottles were sealed with screw caps and heated in a 80 °C water bath 6 hr, held at 4 °C 12 hr and allowed to warm to room temperature (20–23 °C). Turbidity was measured by visual observation and by a Hunter Model D25P-2 Color Difference Meter.

Gel electrophoresis

Lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS PAGE), silver staining, and glycoprotein detection were performed as described by Hsu et al. (1989). All juice samples were filtered through 0.45 μm membrane before gel electrophoresis.

RESULTS & DISCUSSION

Effect on turbidity and browning

The effect of variety, maturity and processing on turbidity is shown in Table 1. Pre-filtration processing had similar effects on turbidity regardless of whether the fruit was ripened or not. All juices were clear after fining and filtration. The apparent high turbidity in the enzyme treated juices resulted from resuspension of settled solids before turbidity determination. Only a trace of haze developed in bottled juices after pasteurization. In contrast, moderate to strong haze was observed in reconstituted single strength juices (SSJ). Our results indicated concentration had a more severe effect than pasteurization on juice haze formation. This may be due to increases in concentration of reactants (eg., proteins, phenolics, polysaccharides, etc.) which form insoluble complexes and become less soluble during reconstitution. Processing with or without SO_2 had no effect on turbidity.

Browning was reduced by processing with SO_2 which may act as an inhibitor to prevent both enzymatic and non-enzymatic reactions. Juices were extremely cloudy before fining and filtration, so degree of browning could not be measured before this step. Table 2 shows that Bartlett variety generally had the highest degree of browning followed by Comice and then d'Anjou under the same conditions.

In general, the higher pH favored enzymatic browning or Maillard reaction. Bartlett had a higher pH value than Comice and d'Anjou (pH 4.4 vs. pH 4.0 and 3.9, respectively). In addition, formol value of these concentrates reported elsewhere (Wrolstad et al., 1989) indicated Bartlett had higher free amino acids than the other two. These may explain why Bartlett had a higher degree of browning. Degree of browning in reconstituted SSJ was higher than in bottled juices. As with tubidity, concentration had a more severe effect on browning than pasteurization. This may be due to a similar effect as in turbidity, increased concentration of reactants enhanced the Maillard browning reactions.

Proteins and heat stability

An increase in total proteins in press juices was observed with increased fruit maturity (except Bartlett) and with processing using SO_2 (Table 3). In general, high temperature short time heat treatment (HTST) increased the total soluble proteins in the juice samples. Similar results were observed in apple juices (Hsu et al., 1989). This may be from extraction of proteins from cell walls, or from heat induced dissociation of protein-phenolic complexes releasing more detectable proteins, or from heat-unfolding of protein molecules exposing more detectable reactive sites.

Fining and filtration reduced the concentration of proteins in the order of 5 to 23 mg/L depending on variety, maturity and processing condition. About 5 to 20 mg/L of proteins still remained in the juice samples. Hsu and Heatherbell (1987a) indicated that relatively large amounts of bentonite were re-

Table 3—Effect of variety, maturity and processing on concentration of soluble protein[a] in pear juices using a modified Bio-Rad procedure

Juice samples[b]	Press juice	HTST-treated	Enzyme-treated	Fined/filtered	Bottled	Reconstituted
d'Anjou						
Hard green ($-SO_2$)	6.8 ± 0.2	11.1 ± 1.3	11.9 ± 0.2	7.0 ± 0.4	0.5 ± 0.3	7.9 ± 0.1
Hard green ($+SO_2$)	11.9 ± 0.2	27.5 ± 0.9	33.3 ± 0.1	10.7 ± 0.3	5.1 ± 0.4	15.8 ± 1.2
Soft ripen ($-SO_2$)	19.2 ± 0.5	36.5 ± 0.2	10.2 ± 1.2	5.4 ± 0.2	0	6.0 ± 0.1
Comice						
Hard green ($-SO_2$)	12.2 ± 0.5	27.4 ± 0.4	24.6 ± 0.4	5.2 ± 0.4	0.1 ± 0.1	6.1 ± 0.3
Hard green ($+SO_2$)	22.1 ± 0.5	52.5 ± 1.4	42.1 ± 0.2	20.3 ± 0.1	18.5 ± 0.5	23.7 ± 0.6
Soft ripen ($-SO_2$)	45.0 ± 0.4	46.6 ± 0.9	18.9 ± 2.6	10.6 ± 0.1	6.4 ± 0.8	10.4 ± 0.4
Bartlett						
Hard green ($-SO_2$)	31.1 ± 0.6	23.3 ± 0.5	16.1 ± 0.7	8.9 ± 1.7	6.5 ± 1.0	14.0 ± 3.4
Soft ripen ($-SO_2$)	29.6 ± 0.5	37.5 ± 0.6	27.8 ± 1.6	10.2 ± 0.5	4.2 ± 0.4	11.7 ± 0.3

[a] Values are mean ± SEM for n=2 processing trials, mg/L.
[b] Refer to Fig. 1.

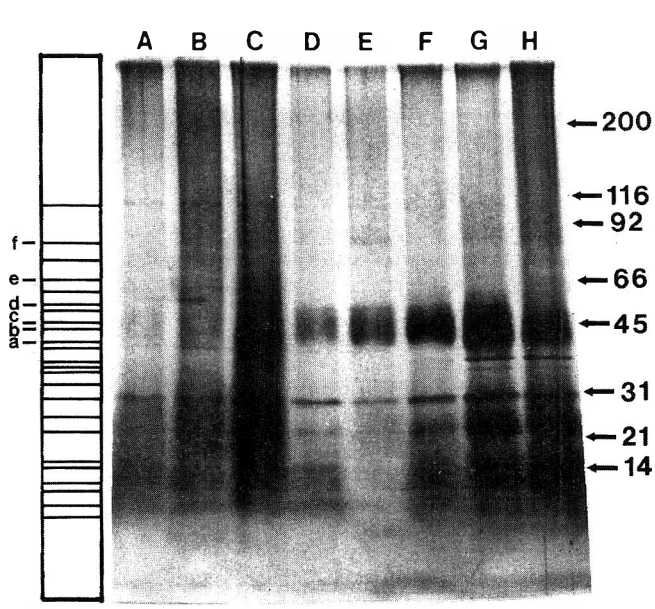

Fig. 2.—LDS PAGE of pear juice proteins: Effect of variety, maturity, and processing (± SO_2). (A) Comice (hard green, $-SO_2$, pH 4.05). (B) Comice (hard green, $+SO_2$, pH 4.05). (C) Comice (soft ripened, $-SO_2$, pH 4.01). (D) d'Anjou (hard green, $-SO_2$, pH 3.96). (E) d'Anjou (hard green, $+SO_2$, pH 3.94). (F) d'Anjou (soft ripened, $-SO_2$, pH 3.83). (G) Bartlett (hard green, $-SO_2$, pH 4.40). (H) Bartlett (soft ripened, $-SO_2$, pH 4.38). Eighty μL of each sample was applied to gel. Molecular weights (daltons × 10^{-3}) of standards are on the right.

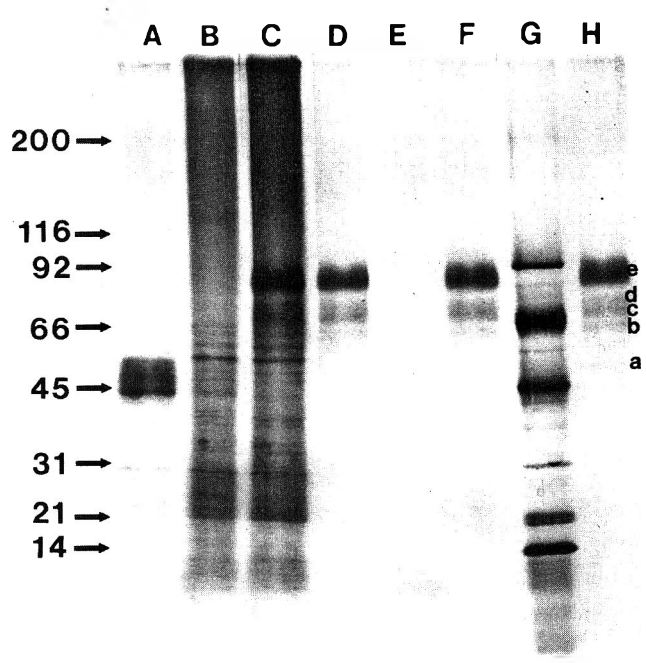

Fig. 3.—LDS PAGE of d'Anjou juice proteins (hard green, $+SO_2$): Effect of processing. (A) Press juice. (B) HTST-treated juice. (C) Enzyme-treated juice. (D) Fined/filtered juice. (E) Bottled juice. (F) Reconstituted juice. (G) Molecular weight standards. (H) Enzyme solution. Eighty μL of each sample was applied to gel. Molecular weights (daltons × 10^{-3}) of standards are on the left.

Table 4—Effect of variety, maturity and processing on Hunter turbidity reading[a] and visible turbidity[b] of pear juices after heat test

Juice samples[c]	Bottled	Reconstituted
d'Anjou		
Hard green ($-SO_2$)	0 (−)	0.9 (+ −)
Hard green ($+SO_2$)	0 (−)	0.8 (+ −)
Soft ripen ($-SO_2$)	0 (−)	1.2 (+ −)
Comice		
Hard green ($-SO_2$)	0 (−)	0.2 (−)
Hard green ($+SO_2$)	0.1 (−)	0.8 (+ −)
Soft ripen ($-SO_2$)	0 (−)	2.4 (+ −)
Bartlett		
Hard green ($-SO_2$)	0.4 (−)	2.5 (+ −)
Soft ripen ($-SO_2$)	0.1 (−)	2.1 (+ −)

[a] Values are means of duplicate processing trials.
[b] Turbidity of visible observation indicated in brackets ( ): (−) = Clear; (+ −) = Trace Haze (only detected under strong light); (+) = Slight Haze; (+ +) = Moderate Haze; (+ + +) = Strong Haze; (+ + + +) = Extreme Haze.
[c] Refer to Fig. 1.

quired to remove the proteins which are the most resistant to fining.

Second pasteurization at bottling reduced the final juice protein concentration. This may be due to heat denaturation of proteins and their subsequent precipitation (bottled juices were filtered before protein determination, refer to Materials and Methods). Consequently, traces of haze were detected in bottled juices (Table 1). Concentration had no effect on the juice protein content (Table 3). However, reconstituted SSJ had a higher degree of browning/oxidation than bottled juices (Table 2). Soluble proteins which remain in reconstituted SSJ may become insoluble during heat testing. This may explain why reconstituted SSJ are less heat stable than bottled juices (Table 4).

Characterization of soluble proteins

To further understand the action of proteins among different varieties (Comice, d'Anjou and Bartlett) and during maturation and processing, lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS PAGE) and protein blotting for glycoprotein detection were used to characterize the individual protein fractions. Although there were electrophoretic differences among three varieties, maturation had a similar effect on the concentration of the protein fractions present. As shown in Fig. 2, ripened fruits not only had higher protein concentration, but also had more protein fractions than unripened ones. Up to 24 protein fractions with MW in the range of 11,400 to 112,000 daltons were detected in press juices. Six protein fractions containing glycoproteins were detected in Bartlett (Fig. 2, frac-

tions a,b,c,d,e,f), and three in d'Anjou and Comice (Fig. 2, fractions a,b,c).

Effect of processing on protein fractions showed a similar trend among varieties. d'Anjou (hard green, $+SO_2$) was selected to demonstrate that protein fractions changed during processing (Fig. 3). HTST treatment increased the total protein concentration (Table 3) and protein fractions (Fig. 3(B)) in the juices. No protein band was detected in bottled juices (Fig. 3(E)), however 5.1 mg/L of protein was determined in same sample by a modified Bio-Rad method (Table 3). The Bio-Rad dye binding method detected the proteins and polypeptides with MW greater than 3,000 daltons (Sedmak and Grossberg, 1977), but gel electrophoresis detected the protein with MW greater than 10,000 daltons (Fig. 3). The majority of proteins remained in bottled juices could be the low MW proteins/polypeptides with MW in the range between 3,000 and 10,000 which were not detected on the electrophoretogram.

Protein fractions with MW in the range of 64,000 to 92,000 daltons, mainly glycoproteins, were detected in enzyme clarified juices (Fig. 3(C)). These fractions were neither found in press juices nor HTST treated juices, were not removed by fining or filtration (Fig. 3(D)), and remained in the reconstituted SSJ (Fig. 3(F)). They were removed only during the second pasteurization before bottling and were not detected in bottled juices (Fig. 3(E), bottled juices were filtered before electrophoresis). Similar effects were found in the d'Anjou (unripened and ripened) processed without SO_2 and other varieties (data not shown). The results indicate these fractions were sensitive to heat and detected after enzyme clarification. They may originate from the enzyme preparation, or the extraction or interaction of pear proteins during enzyme clarification.

To further understand the source of these fractions (64,000–92,000), the enzyme solutions, with the same concentrations added to juices during clarification, were subjected to LDS PAGE. As shown in Figure 3(H), five protein fractions (a,b,c;d,e) were detected. Fractions b, c, d, and e (Fig. 3(H)) with MW in the range of 64,000 to 92,000 containing glycoproteins appeared to be the major protein fractions in the enzyme solution. These results suggest that (1) proteins found in the enzyme preparation may remain in juice products with the potential to contribute to post-clarification haze and sediment formation, and (2) haze and sediment from unstable enzyme proteins can be prevented by heat denaturation (pasteurization) and precipitation of these proteins before final filtration and bottling.

In contrast to a similar study on apple juice (Hsu et al., 1989), no pear protein fractions were detected in juices after fining and filtration (Fig. 3(D)); only the protein fraction from the clarifying enzyme preparation was detected at that stage. The results illustrate that the proteins in different fruits can react quite differently upon processing, and some enzymes added for clarification during processing have the potential to contribute to post-clarification haze and sediment formation.

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A Rapid Method for Separation and Quantitation of D-Malic Acid in Fruit Juice

T.A. EISELE and J.R. HEUSER

ABSTRACT

A HPLC method using an aqueous mobile phase containing the chiral ligand-exchanger Cu II-L-valine complex at pH 5.5 with a polystyrene divinyl-benzene copolymer column was used to resolve D-malic acid in apple, pear, and Concord grape juices. D-malic acid was detected and quantitated at 330 nm in less than 15 min per sample. The detection limit appeared to be 2 mg/100 mL D-malic acid in 12 Brix juice, or 0.33% total malic acid in a typical apple juice containing 0.6 g/100 mL using the described procedure.

INTRODUCTION

SOME INVESTIGATIONS to verify authenticity of apple juice products have been directed toward nonvolatile acid analysis and detecting adulterative addition of synthetic DL-malic acid (Lee and Wrolstad, 1987). Since cost of pure L-malic acid, the naturally occurring form, precludes its addition, efforts have been directed to detecting D-malic acid. L-malic acid can be quantitated by enzymatic procedures (Mollering, 1974) and DL-malic acid by HPLC (Coppola and Starr, 1986). The presence of the D-enantiomer is indirectly determined by difference (Evans et al., 1983). An inter-laboratory study (Zyren and Elkins, 1985) showed that the precision of this procedure was good; however, synthetic DL-malic acid had to be added to the 20% level before the apple juice could be classified as adulterated at a 95% confidence levels.

Several recent reports (Benecke, 1984; Horikawa et al., 1986; Heuser, 1988) have shown that enantiomeric α-hydroxy acids, which includes D- and L-malic acids, were efficiently resolved by ligand exchange HPLC. The technique uses a reverse-phase column with a chiral mobile phase consisting of a complex of Cu II and an amino acid such as L-valine (Armstrong, 1987; Gil-Av et al., 1980) or an amino acid derivative such as N,N-dimethyl-L-valine (Doner and Cavender, 1988). After chromatographic separation, the α-hydroxy acids were then complexed with Fe III in a post column reaction coil and detected at about 420 nm. The test did not need to be quantitative, since presence of any D-isomer would indicate adulteration.

In another recent study (Agarwal, 1988), a gas chromatographic method was developed for simultaneous determination of D- and L-malic acids in apple juice. The (R)-(-)-2-butanol esters were prepared and the diastereomers were separated and quantitated on a Carbowax 20M fused silica capillary column. The procedure did not involve extraction or cleanup step; however, considerable time (1 hr) was required to prepare the derivatized ester.

There were two purposes for this study: to demonstrate that D-malic can be quantitated by using a simplified variant procedure of the HPLC chiral liquid chromatographic method thus eliminating the need for a post column reaction system and reducing the required analysis time to less than 15 min per sample; and to demonstrate that the described chromatographic method applies to detection of D-malic acid in pear juice and Concord grape juice as well as apple juice.

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MATERIALS & METHODS

Reagents

Authentic juices were prepared to 12° Brix from commercial samples of concentrates (Tree Top Inc) produced from Red Delicious apples, Bartlett pears, and Concord grapes. Samples were filtered through 0.45 u membrane prior to HPLC injection. The L-valine and the D- and L-enantiomers of malic acid were purchased from Sigma Chemical Co (St. Louis, MO).

HPLC procedures

The HPLC system consisted of a Waters 510 pump, and a 710B Wisp autosampler and two detectors. The first detector was a Waters 990 photodiode array detector used to acquire the various spectra, and the second detector was a Waters 490 programmable variable wavelength detector used for routine analysis. The 990 photodiode detector was set at a resolution of 2 nm with a scanning time of 5 seconds from 290 to 450 nm. It was interfaced to a NEC PowerMate 386/20 computer using the Waters 990+ photodiode array software program. The Waters 490 detector was set at a wavelength of 330 nm at 1.0 AUFS. It was interfaced to a Nelson 760 series buffer and an IBM XT using the Nelson 3000 series Chromatography Data System software program. The column, a PLRP-S 5u (polystyrene divinyl-benzene copolymer), 25 cm × 4.6 mm from Polymer Laboratories, Inc. (available from Alltech Associates, Inc., Deerfield, IL), was used with a mobile phase consisting of 16mM L-valine and 8 mM copper acetate adjusted to pH 5.5 with NaOH in water at 1.2 mL/min. All injection volumes were 10 μL.

Standards

D- and L-malic acid standards were prepared over the range of 0.0–200.0 mg/100 mL each. The same amounts of D-malic were added to 12° Brix apple juice, pear juice, and Concord grape juice. The linear correlation coefficients were calculated using least squares analysis of the area of the D-malic acid peak at 330 nm vs the various concentrations of D-malic acid.

RESULTS & DISCUSSION

THE ABSORBANCE SPECTRUM from 190 to 450 nm of the mobile phase reagent, the Cu II (L-valine) complex, compared to the absorbance spectrum of water, as the reference, is shown in Fig. 1. For the mobile phase alone, there was a sharp absorbance increase starting at about 350 nm and maximizing at about 290 nm (A). When the mobile phase contained D-malic acid, it was noted that the absorbance spectrum shifted slightly to the higher wavelengths (B). At wavelengths less than 290 nm, the absorbance spectra of the mobile phase alone and with D-malic acid was beyond the limits of the detectors; thus limiting any useful detection to wavelengths greater than 290 nm.

Using the mobile phase alone rather than water as the reference, a characteristic absorbance curve was generated for the mobile phase containing D-malic acid with an absorbance maximum at about 305 nm as shown in Fig. 1 (C). Since the absorbance values were beyond the limit of the photo diode array detector at wavelengths less than 290 nm, an irregular baseline appeared which was due to detector amplification noise. Nevertheless, the absorbance curve demonstrate a range of wavelengths between 300 and 350 nm that could be used to

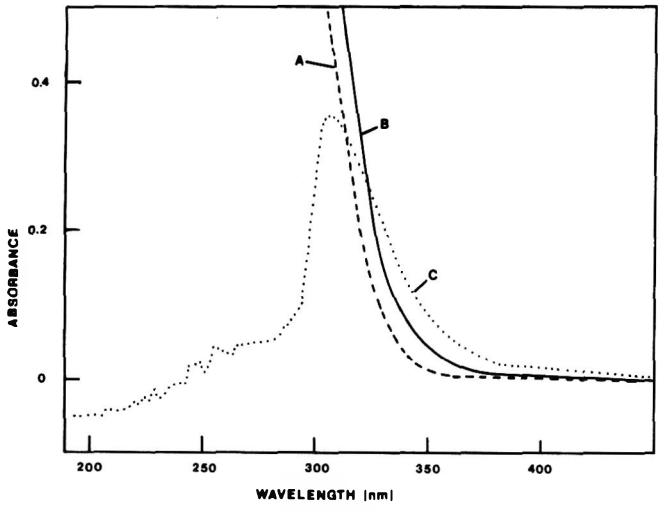

Fig. 1—Absorbance spectra of: the HPLC mobile phase (A) and the mobile phase with D-malic acid (B) using water as the reference; and the mobile phase with D-malic acid (C) using the mobile phase alone as the reference.

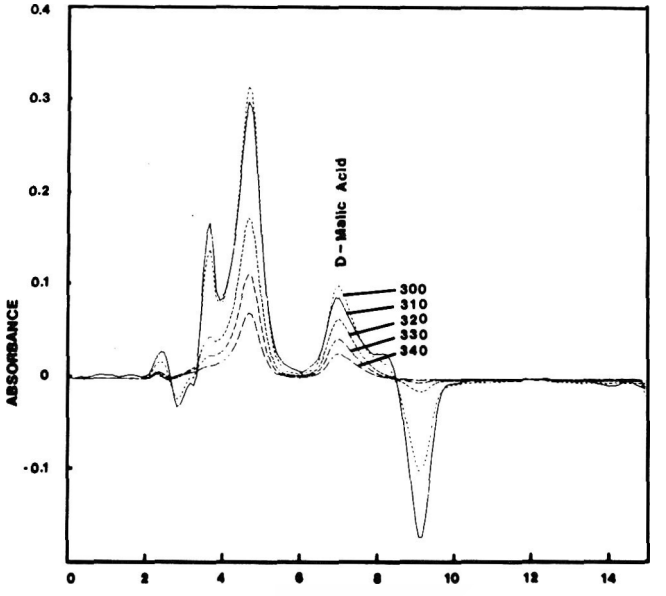

Fig. 2—Absorbance chromatograms of D-malic acid in mobile phase at wavelengths of 300, 310, 320, 330, and 340 nm.

detect D-malic acid in the mobile phase. To determine which wavelength was appropriate for detection of D-malic acid in the mobile phase, absorbance chromatograms were obtained at 300, 310, 320, 330, and 340 nm and plotted on one graph (Fig. 2). The retention time for the elution of L-malic acid was 4.6 min and for D-malic acid, 7.3 min. As expected, the wavelength closest to the maximum absorbance of 305 nm had the highest absorbance for both L- and D-malic acid. Also, a negative peak was noted with all samples shortly after elution of the D-malic acid peak and the size tended to be directly proportionate to the absorbance increase of the positive peak.

The results of the absorbance chromatograms from various concentrations of D-malic acid in the mobile phase are shown in Fig. 3. The retention time of both L- and D-malic acid varied with concentration; an observation also mentioned by Horikawa et al. (1986). As the concentration of D-malic acid decreased, the retention time of the peak increased and migrated toward the negative peak. As a result, integrating the area of a small concentration of D-malic acid at wavelengths less than

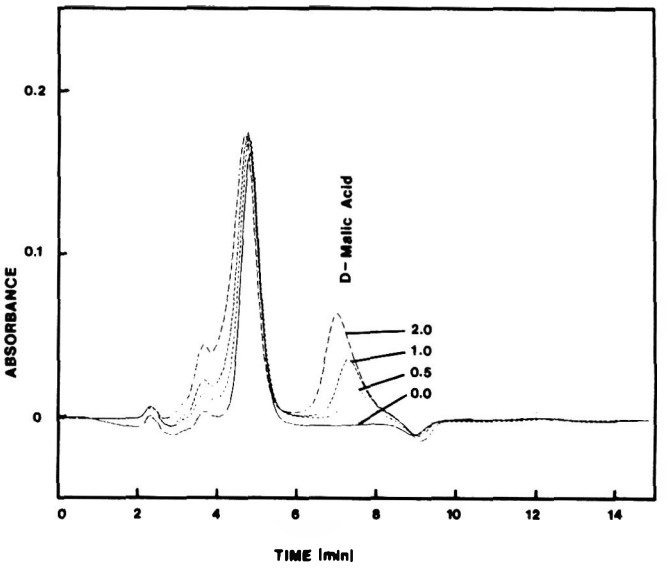

Fig. 3—Absorbance chromatograms at 330 nm of 0, 0.5, 1.0, and 2.0 mg/mL D-malic acid in mobile phase.

330 nm, became more difficult. As a compromise between sensitivity and adequately integration of the peak, the wavelength of 330 nm was chosen as optimum for the two detectors we used.

The correlation coefficients from the linear regression analysis of the area of the D-malic acid peak at an absorbance of 330 nm versus concentrations of D-malic acid in the mobile phase (standard curve), the augmented apple juice, pear juice, and Concord grape juice were between 0.9990 and 0.9999. Recoveries ranged from 98.1 to 100.8% at the concentrations of D-malic acid we used. Examples of absorbance spectra at 330 nm for various juice samples and juice augmented with 0.5 mg/mL of D-malic acid is shown in Fig. 4. In the many authentic samples of apple, pear, and Concord grape juices we tested, no peaks eluted at the same retention time as D-malic acid under the conditions described. Also, at 330 nm, the lower limit of detection for D-malic acid in the various augmented fruit juices appeared to be between 20 and 25 μg/mL juice. In a typical 12° Brix apple juice sample with 0.6 g/100 mL malic acid (Lee and Wrolstad, 1988), a detection limit of 20 μg/ml (2 mg/100ml) would equate to 0.33% D-malic acid.

Horikawa et al. (1986) commented that retention of α-hydroxyacid enantiomers on reverse phase columns was dependent on molar concentrations of the enantiomers as well as eluent concentration of amino acids and pH. In apple juice where the concentration of L-malic acid (0.6 g/100mL) may be in excess of 300 times more than the concentration of D-malic acid (2 mg/100 mL), the capacity of the column to adequately resolve enantiomers may be exceeded. As such, interaction between the column stationary phase and the ternary mixed-ligand complex becomes an extremely important function.

The silica-based ODS, C-8, and C-18 columns failed to adequately resolve the smaller D-malic acid peak from the larger L-malic acid peak in the apple juice matrix or separate the D-malic acid from the "negative" chromatogram peak that followed. The Hamilton polystyrene divinyl-benzene copolymer column did meet this separation criteria.

The quantitation of L-malic acid was not practical in the fruit juice samples because unknown peaks eluted at the same retention time, 4.6 min (Fig. 4). However, by changing from L-valine to D-valine in the mobile phase (Heuser, 1988), the elution order of the two malic isomers should be reversed and L-malic acid could be quantitated using the same procedure.

The procedure we describe successfully separated the malic acid enantiomers in apple, pear, and Concord grape juices such

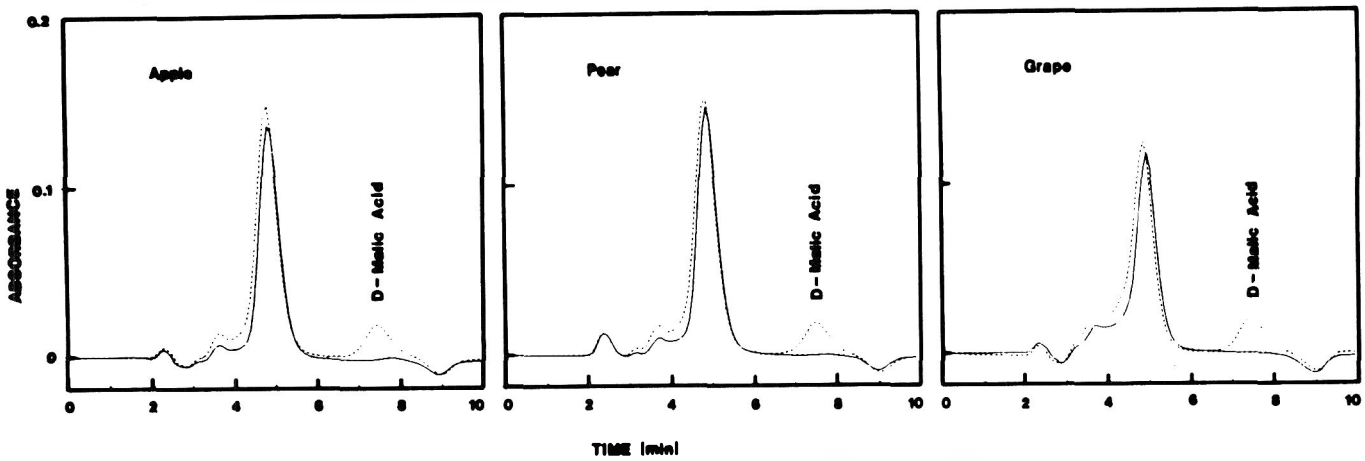

Fig. 4—Absorbance chromatograms at 330 nm of 12° Brix: apple juice plain and with 0.5 mg/mL D-malic acid; of pear juice plain and with 0.5 mg/mL D-malic acid; and of Concord grape juice plain and with 0.5 mg/mL D-malic acid.

that D-malic acid could be resolved and quantitated. The procedure is simple and is less prone to error than the indirect determination of D-malic acid by difference methodology (Evans et al., 1983).

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fermentations of raisin extracts containing higher copper content. In conclusion, increase in the copper uptake by cells caused a decrease in glucose uptake. Accordingly we observed the inhibition in ethanol productivity, yield and cell growth, observed in repeated batch fermentations in presence of relatively high copper levels can be attributed to reduction of glucose uptake rate, caused by increased copper uptake.

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Controlled Atmosphere Storage of Oranges to Enhance Aqueous Essence and Essence Oil

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ABSTRACT

Freshly harvested Valencia and Pineapple oranges were treated 24 hr under anaerobic conditions at room temperature with nitrogen and < 1% oxygen. Juices from 820 kg each of control and treated fruit were concentrated in a pilot scale evaporator to 60–67°Brix and aqueous essences (800–900 mL) and essence oils (60–109 mL) collected. Capillary gas chromatography quantified 26 volatile components in aqueous essence and 23 in essence oil for each sample. Aqueous essences from treated fruit showed increased acetaldehyde, ethanol and several other alcohols, aldehydes and esters, and essence oils from treated fruit showed increased methanol, ethanol, ethyl acetate, and ethyl butyrate. Flavor panels determined significant differences in essences and essence oils from treated vs control Valencia but found no differences in treated vs control Pineapple oranges.

INTRODUCTION

AQUEOUS ORANGE ESSENCE (AE) and essence oil (EO) are commercially important flavor fractions recovered when orange juice is concentrated to produce frozen concentrated orange juice (FCOJ) (Johnson and Vora, 1983). AE is widely used to flavor FCOJ and other products made by reconstitution of FCOJ to single-strength juice. EO is also used to flavor orange products, but has recently been fractionated to provide specific flavor fractions (e.g. ester, aldehyde, green flavor) to add to AE to enhance flavor properties (Hendrix, 1990). Methods for enhancing the desirable volatile components in orange juice before concentration would increase value of AE and EO made from the juice.

Earlier studies have shown that ethanol in fruit increased after anaerobic treatment of oranges 20 hr at 32°C (Bruemmer and Roe, 1969) and in grapefruit after treatment 16–32 hr at 37°C (Bruemmer and Roe, 1970). Davis et al. (1973) found increases in ethanol and acetaldehyde in oranges and grapefruit stored several weeks in atmospheres low in oxygen and high in carbon dioxide. Changes in other volatile components were not determined in those studies. Earlier studies at our laboratory on small quantities of Hamlin, Pineapple and Valencia oranges (18 kg) showed that anaerobic storage in N_2 or CO_2 caused rapid increases in several volatile components (Shaw et al., 1990).

The objective of our current study was to monitor rapid changes in more than 20 components of orange AE and EO after anaerobic treatment of freshly harvested fruit and to relate changes in flavor and aroma of AE and EO using sensory panels. We studied the potential for increasing strength of commercial EO and especially AE by short-term storage of oranges under controlled atmosphere before processing.

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MATERIALS & METHODS

Fruit Samples

Forty boxes (1650 kg) were used each of Pineapple oranges harvested Jan. 22, 1989 and Valencia oranges harvested April 9, 1989. Fruit were washed and sized with a standard commercial mechanical sizer. Equal size distribution lots were assembled and placed in four 10-box containers.

Controlled atmosphere treatments

Two 10-box lots of experimental fruit (treated under nitrogen atmosphere) of each harvest were contained in double 300 gal, 3 mil polyethylene bags closed to create air-tight chambers. A piece of 0.635 cm (1/4 in.) o.d. stainless steel tubing 76 cm long was inserted through the top of each bag so that nitrogen gas could be introduced into the bottom of the containers and escape through the top at the closure, sealed with duct tape. Two YS1 model 5331 oxygen probes attached to a model 5300 Biological Oxygen Monitor (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) were inserted, one through each bag at the top of the 10-box fruit sample to monitor oxygen during the controlled atmosphere treatment. Nitrogen was introduced at a high flow rate (> 1200 mL/min) until the oxygen level inside the bag dropped to < 1% (2–3hr). The nitrogen flow was reduced to 1200 mL/min and maintained 24 hr. The oxygen content remained at 0.5–0.9% during treatment at room temperature. Control fruit were stored in open 10-box containers adjacent to the experimental fruit during treatment. Storage temperatures were 18–20°C for Pineapple and 23–26°C for Valencia oranges. The two 10-box lots each of control or treated fruit were combined into a single 20-box lot for processing.

Control and treated fruit were processed on the same day as follows. Fruit were extracted and juice finished with standard commercial methods using an FMC 391 "in-line" extractor and FMC Model 35 finisher, 0.05 cm (0.020 in.) screen discharging pulp with about 190 "quick fiber" (Attaway and Carter, 1975). Juice was concentrated with a three-effect, four-stage temperature accelerated short time evaporation (TASTE) evaporator (Gulf Machinery Corp., Safety Harbor, FL) having 500 lb/hr water removal capacity at the Citrus Research and Education Center, Lake Alfred, FL (Bates and Carter, 1984; Nagy and Shaw, 1980). Concentrated juice, AE and EO were collected (Table 1).

Gas chromatography (GC)

AE and EO were analyzed on a Hewlett-Packard Model 5880A GC equipped with a 0.32 mm i.d. × 50m capillary fused silica cross-linked 5% phenylmethyl silicone column (Hewlett-Packard, Avondale, PA) and a capillary inlet system fitted with a 100:1 splitter. Helium flow was 1.5 mL/min and injection port and detector were 275°C. The column was 60°C for 4 min, then increased to 200°C at 6°C/min and held for 15 min. AE (1.0 μL) and EO (0.2 μL) and standard calibration mixtures for each were injected manually. The internal standard was acetonyl acetone. Acetaldehyde and methanol were quantified in AE using a Hewlett-Packard Model 5890 GC equipped with a 0.32 mm i.d. × 60m capillary fused silica DB Wax column (J & W Scientific, Folsom, CA) and other instrument parameters as described above for the Model 5880A GC. The column was held at 70°C. Peak areas were used for quantification. Standard mixtures of components quantified in AE were prepared in water (Table 2), and of those in EO were prepared in limonene (Table 3). Six runs of each standard and AE were averaged to determine amounts shown in Table 2, and four runs of standard and EO were averaged for the values reported in Table 3. The coefficient of variation for AE and EO components was generally less than 18% (internal standard method).

Table 1—Products from TASTE evaporator

Cultivar	Juice: Starting[a] Gal	°Brix	Acid %	Ratio °B/% Acid	Oil % × 10^{-2}	Concentrate Gal	°Brix	Aqueous essence mL	%EtOH[b]	Essence Oil (mL)
Pineapple										
control	113	11.71	1.05	11.15	3.6	10.0	62.2	900	8.25	97
treated	117.5	11.49	0.97	12.35	3.5	10.3	61.8	905	17.0	109
Valencia										
control	105	12.38	0.88	14.06	2.6	15.5	67	771	8.75	61
treated	112	12.37	0.87	14.22	2.6	18.0	62.5	780	18.0	60

[a] From 820 kg of fruit
[b] Determined by hydrometer.

Table 2—Quantities (ppm) of volatile components in aqueous essences from controlled atmosphere treated and control Pineapple and Valencia oranges

Component	Pineapple cultivar Control	Pineapple cultivar Treated	Valencia cultivar Control	Valencia cultivar Treated
Acetaldehyde[a]	1760	3380[b]	1930	4960[b]
Methanol[a]	2660	3800[b]	2900	3930[b]
Ethanol	81,000	160,000[b]	89,000	175,000[b]
Acetone	1.5	1.7	23	29
1-Propanol	2.9	4.7[b]	40	82[b]
Ethyl acetate	11	20[b]	75	135[b]
Isobutanol	0.8	1.4[b]	12	29[b]
1-Butanol	0.8	3.9[b]	2	20[b]
1-Penten-3-ol	0.66	0.78[b]	11	14[b]
1-Penten-3-one	1.6	2.3[b]	12	16[b]
Methyl butyrate	0.34	0.40[b]	3.9	5.2[b]
1,1-Diethoxyethane	tr	tr	9	127[b]
3-Methyl-1-butanol	1.9	3.3[b]	31	74[b]
2-Methyl-1-butanol	0.27	0.41[b]	5	11[b]
Ethyl butyrate	10	21[b]	78	185[b]
trans-2-Hexenal	5.7	6.6[b]	52	53
trans-2-Hexenol	0.68	0.81[b]	15	15
Octanal	0.85	1.06[b]	6.5	11[b]
Octanol	0.57	0.85[b]	10.2	12.4[b]
Linalool	4.7	5.5[b]	40	46
Ethyl-3-hydroxyhexanoate	3.6	3.2[b]	13	13
Terpinen-4-ol	0.4	0.4	3	3
α-Terpineol	0.8	0.7	5.0	4.7
Neral	0.2	0.3[b]	1.9	2.7[b]
Geranial	0.2	0.3[b]	1.7	2.2
Valencene	0.57	0.22[b]	1.6	3.8[b]

[a] Values determined on a polar DBWAX column.
[b] Significantly different from control at a 95% confidence level or greater. The coefficient of variation for all values was less than 18%.

Table 3—Quantities (ppm) of volatile components in essence oils from controlled atmosphere treated and control Pineapple and Valencia oranges

Component	Pineapple cultivar Control	Pineapple cultivar Treated	Valencia cultivar Control	Valencia cultivar Treated
Methanol	92	270[a]	110	360[a]
Ethanol	1200	2600[a]	1600	3300[a]
Ethyl acetate	210	510[a]	80	180[a]
1-Penten-3-one	81	96[a]	30	30
Methyl butyrate	97	95	59	62
Ethyl butyrate	2800	6000[a]	2500	6300[a]
trans-2-Hexenal	990	1080[a]	290	270
trans-2-Hexenol	31	37[a]	76	40
α-Pinene	5200	5100[a]	4700	4600
Sabinene	2900	3200[a]	2500	2400
Myrcene	30,900	30,800[a]	28,900	28,300
Octanal	5570	5160[a]	4080	3940
Octanol	150	180[a]	93	77
Linalool	4869	4930[a]	2800	2600
Nonanal	790	750	600	600
Ethyl 3-hydroxyhexanoate	58	56	27	23
Citronellal	590	630[a]	420	450
Decanal	3570	3530	3440	3180
Neral	850	960	730	590
Carvone	470	480	630	640
Geranial	1150	1190[a]	740	600[a]
Perillaldehyde	230	220	220	200
Valencene	19,200	20,300	25,500	24,900

[a] Significantly different from control at 95% confidence level or greater. The coefficient of variation for all values was less than 18%.

Table 4—Aroma and flavor panel results for Pineapple and Valencia aqueous essences and essence oils

Panel	Strength	Triangle tests results (24 judgments) No. correct	Conf. level (%)
Aroma			
Aqueous essence			
Pineapple	neat	8	N.S.[a]
Valencia	neat	8	N.S.
Essence oil			
Pineapple	neat	7	N.S.
Valencia	neat	12	N.S.
Flavor			
Aqueous essence[b]	mL/1000mL		
Pineapple	control / treated		
equal amounts	2.88 / 2.88	7	N.S.
2 × control	6.06 / 2.88	10	N.S.
Valencia			
equal amounts	2.78 / 2.78	13	95
2 × control	5.70 / 2.78	11	N.S.
	%oil		
Essence oil	control / treated		
Pineapple	0.014 / 0.016	8	N.S.
Valencia	0.018 / 0.020	13	95

[a] N.S. = not significant at the 95% confidence level
[b] Aqueous essence added to 67°Brix Valencia concentrate (control sample) and diluted to 11.8°Brix juice for evaluation.

Sensory evaluations

Triangle difference tests were used for all samples, with 12 experienced panel members provided two sets of samples each for 24 total judgments (Boggs and Hanson, 1949). With oil samples, a 20-minute period lapsed between the two samples to minimize fatigue. For the aroma panels, AE and EO were evaluated neat, 0.6 ml each, in identical one dram screw-cap vials. For all flavor panels, control Valencia orange concentrate (67°Brix "evaporator pumpout") was used as a bland orange juice base. For evaluating AE, the concentrate was diluted to 11.8°Brix and the appropriate amount of AE added, based on alcohol content of the treated sample as indicated in Table 4 (J.D. Johnson, personal communication). A second evaluation was carried out with the weaker control AE added at twice the original level vs. treated AE at the original level.

For sensory evaluation of EO in juice, a small sample of concentrated juice with high oil was prepared for blending to the desired final oil level. Thus, 1.7 mL of EO was added to concentrate dropwise with magnetic stirring over 5 min to provide 0.11% oil in single strength juice, determined by the Scott oil method (Scott and Veldhuis, 1966). This orange concentrate (31g) with high oil was then added to 182g original concentrate so the final oil level of juice from the blended concentrate would be ca. 0.017%. The values for oil levels in the juice samples were recorded (Table 4).

RESULTS & DISCUSSION

Aqueous essences

The anaerobic 24-hr treatment almost doubled the content of the major constituent, ethanol, in AE from both cultivars (Table 2). The percent ethanol in Table 1 determined with a hydrometer were in close agreement with those in Table 2

determined by GC (% EtOH × 10^4 = ppm EtOH). Most other alcohols in AE were also increased by the anaerobic treatment. Two alcohols, terpinene-4-ol and α-terpineol, were not affected by the treatment, while *trans*-2-hexenol and octanol increased only in the Pineapple cultivar.

Four esters were quantified. Amounts of ethyl acetate and ethyl butyrate were almost doubled by the anaerobic treatment, while methyl butyrate was less affected and ethyl 3-hydroxyhexanoate was practically unchanged by the treatment. Ethyl butyrate is considered important to orange juice flavor (Ahmed et al., 1978). While the role of other esters in orange flavor is unclear, the total esters in orange AE has been used as an indication of quality (Attaway et al., 1967). The four aldehydes measured, acetaldehyde, octanal, neral and geranial are considered important to orange flavor (Ahmed et al., 1978). Levels of these aldehydes showed significant increases in treated samples except geranial, which was unchanged in AE from Valencia. Another component which increased in treated Valencia orange AE was 1,1-diethoxyethane. This acetal is considered an artifact formed from ethanol and acetaldehyde during processing in the acidic juice medium (Nursten, 1970). Its increase in treated Valencia AE with higher acetaldehyde and ethanol was not surprising.

Most components were found in control Valencia AE in considerably higher amounts than in control Pineapple AE. The only previous report addressing this point was by Wolford et al. (1968). They used chemical oxygen demand to show that about twice the level of total organics was in Valencia AE as in Pineapple orange AE.

When neat samples of AE were evaluated by aroma panel, no differences between treated and controls from Pineapples or Valencias were found. When AEs were added to bland orange juice base for flavor evaluation, a flavor difference was noted between equal amounts of AEs from controls and treated Valencias. When the level of AE added from control fruit was double that from treated fruit so that the total ethanol was almost equal for the two samples, no significant flavor differences were found.

Essence oils

When quantitative composition of EO from control and treated Pineapple or Valencia juices were compared differences were found similar to those reported above for aqueous essences (Table 3). Levels of ethanol, ethyl acetate and ethyl butyrate were almost doubled and methanol was tripled in EO from treated Pineapple and Valencia compared to EO from controls. Only minor differences were found in other components between treated vs control samples, mainly in EO oil from Pineapple. Many components quantified in EO were different from those in AE because EO is enriched in oil-soluble components present in both juice oil and peel oil (Shaw, 1986). From these results, water soluble components are clearly those primarily affected by anaerobic conditions we used.

When aroma panels evaluated neat samples of EO from controls and treated fruit, no differences were found (Table 4). When EO from control and treated fruit were added to a bland concentrated orange juice base, a flavor difference was noted between oils from control and treated Valencia oranges.

CONCLUSIONS

VALENCIA or Pineapple oranges treated under anaerobic conditions afforded AE and EO flavor fractions with enhanced (almost 2×) amounts of certain alcohols, esters and aldehydes. More changes were noted in water-soluble components in AE than in the oil-soluble components in EO. The flavors of AE and EO from treated Valencias were significantly different from comparable flavor fractions from controls evaluated in bland orange juice base. Such anaerobic treatment has potential to increase strength of commercially important flavor fractions in orange AE and EO.

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Apple Juice Clarification using Mineral Membranes: Fouling Control by Backwashing and Pulsating Flow

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ABSTRACT

New mineral membranes of ceramic (Ceraflo) and carbon (Carbone Lorraine), were used for apple juice clarification using cross flow microfiltration. Effect on performance of the parameters transmembrane pressure, inlet flow velocity, membrane nature, and temperature were studied. Optimum permeate flux was at a transmembrane pressure of about 3.5 bar for both membranes. Formation of a concentration layer of rejected particles was reduced by using techniques backwashing and pulsating inlet flow. These techniques provided a major flux restoration and steady state permeate flux increased by 30–50% with backwash and up to 100% with pulsating inlet flow.

INTRODUCTION

APPLICATION of crossflow microfiltration using mineral membranes (3rd generation membranes), for clarification or concentration processes is comparatively recent in the food industry (Cheryan, 1986; Veyre, 1984; Galaj et al., 1984). These membranes have high adaptability to fluids of different pH, and high temperatures in comparison to polymeric membranes (2nd generation membranes), made of polysulfone, polyamide and polypropylene (Rao et al., 1983; Klein, 1982). Other advantages of clarification by membranes over classical clarification techniques using kieselghur is that final sterile product of required composition is obtained in one step and process time can be considerably shortened. For example, the classical treatment required about 28 hr to obtain clear juice from raw apple juice, while by ultrafiltration, the process time was about 2 hr (Vrignaud, 1983).

In separation processes, the filtration rate is limited either by concentration polarization of rejected solutes near the membrane surface, or by membrane fouling (Bauser et al, 1986), which results in a decrease of permeate flux with time. Conventional methods for increasing flux are use of high inlet velocity or increasing temperature of inlet fluid. These are limited by installation capacity or by quality required of the final product. Other techniques can be used such as pretreatment of membrane surface with substances which control adsorption of rejected particles (Errede, 1984), backwashing (Galaj et al., 1984, Ben Amar and Jaffrin, 1989) or superimposing pulsations of different frequencies on inlet flow (Ben Amar et al., 1987; Keil and Baird, 1971), and intermittent on-off operation which result in dissolving of the concentration polarization layer (Chiang and Zu, 1987, Ben Amar et al., 1989).

The objectives of our study were: (1) to determine effects of temperature, inlet velocity, time, transmembrane pressure and pectinase treatment on apple juice permeate flux using two types of mineral membranes; ceramic on ceramic support or carbon on carbon support, and (2) to investigate methods of reducing or controlling membrane fouling using backwashing or by superimposing pulsations on inlet flow.

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MATERIALS & METHODS

Apple juice

Raw apple juice was obtained directly after apples were pressed and frozen for storage at −15°C. The juice was provided by the Pernod Society, Creteil, France. Initial pH was 3.4 to 3.6 and turbidity was more than 200 NTU (Nephelometric Turbidity Unit). Experiments were conducted with about 15L of fresh apple juice at 50–55°C. To reduce viscosity and pectin content, the apple juice was treated (in some experiments) with pectinase (Enzyme preparation NOVO, 3XL), at a concentration of 0.04% and mixed continuously for 2 hr at 50°C (Thomas et al., 1987).

Mineral membranes

Two types mineral membranes were used: **Ceramic membranes** (Ceraflo) pore size 0.2 μm, composed of 8 tubes 0.28 cm inner diameter and 36 cm long. Total surface area 0.0256 m^2. **Carbon membranes** (Carbone Lorraine) of 6 mm inner diameter and 36 cm long, area 0.0068 m^2 for each single tube. Average pore size 0.2 μm.

Clarification experiments were conducted on two different test benches. The first bench (Fig. 1) is built of stainless steel with two ceramic membranes connected in series fed by a volumetric pump with a maximum flow rate of 30 L/min. This bench included a backwash system which periodically pressurized the permeate circuit at a maximum of 8 bar during short bursts (2 to 5 sec). The back filtered permeate volume was of the order of 10 -20 ml at each burst. The second bench, constructed of PVC in our laboratory, included a 15L tank and a volumetric pump with a maximum flow rate of 10 L/min (Fig. 2). A pneumatically driven pulsation system was inserted between the pump and membrane inlet to superimpose pressure and flow pulsations. The feed flow rate was measured with an electromagnetic flow meter Gould Spectramed (Gould Electronics, U.S.A.) while pressures at inlet and outlet membranes were measured by Validyne pressure transducers (Validyne Engineering Corporation, U.S.A.). The transmembrane pressure was adjusted with a valve downstream of the membrane. The permeate flux was measured with a graduated cylinder and stop watch.

Before each experiment, membranes were cleaned with a basic/acid wash according to procedure provided by manufacturer. The filtration rate of demineralised water at 20°C, expressed in $L/h \cdot m^2 \cdot bar$ was measured before each experiment. The apple juice, treated or not treated with pectinase, was circulated by a volumetric pump through the membranes at 50–55°C. The permeate and retentate were returned to the feed tank to maintain constant inlet concentration. A fresh supply of raw apple juice was used for each run.

Turbidity

Turbidity of clear apple juice was determined with a Nephelometer (Hach Europe, Belgium) in NTU units.

RESULTS & DISCUSSION

Effect of transmembrane pressure

Permeate flux measurements were made at constant inlet velocity of 3 m/sec with both Ceraflo and Carbone Lorraine membranes at different transmembrane pressures (P_{tm}) as shown in Fig. 3a and 3b. The permeate flux increased with the increase in transmembrane pressure up to about 3.5 bar and then became independant of P_{tm}.

Both types of membranes provided about 110–120 $L/h.m^2$ permeate flux at an inlet velocity of 3 m/s and a P_{tm} of 3.5

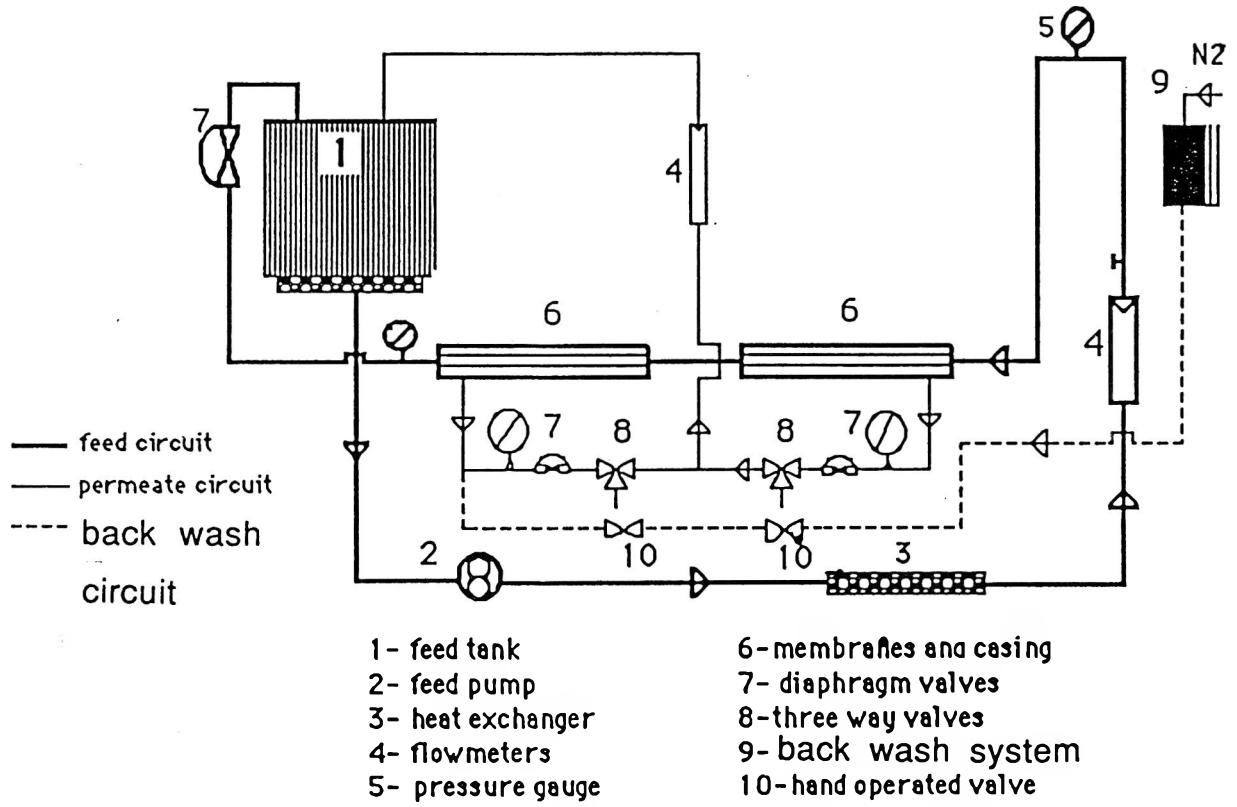

Fig. 1—Schematic of test bench of stainless steel with back pulse system.

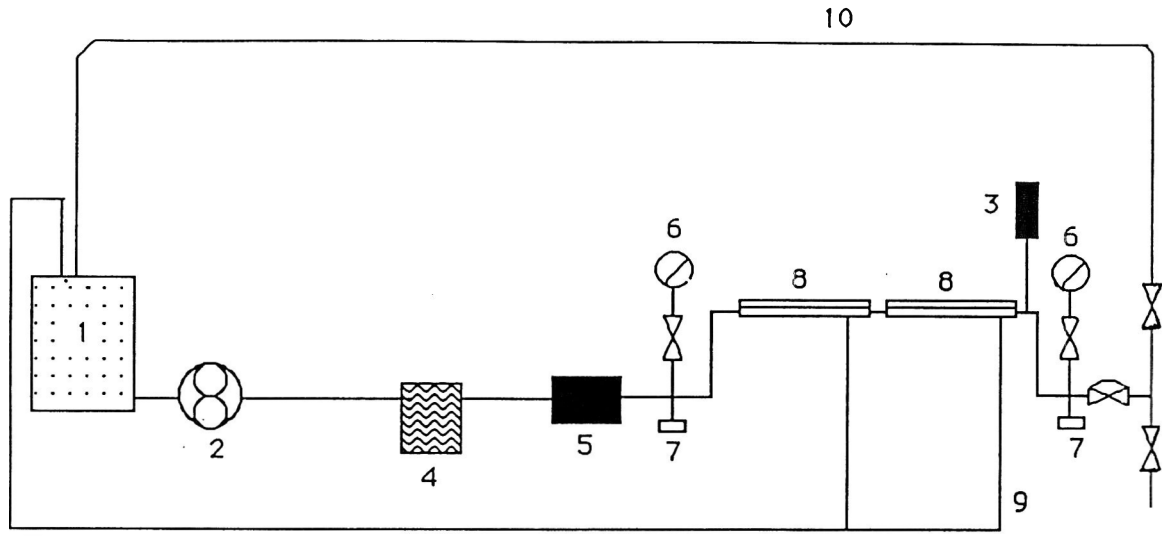

Fig. 2—Schematic of test bench in PVC with pulsating system.

bar. Permeate flux variation with time when transmembrane pressure increased either progressively or instantly is shown in Fig. 4. The permeate flux dropped rapidly during the first 30 min and then stabilised itself. When the transmembrane pressure was increased progressively from 1.4 to 3.5 bar, the permeate flux was lower i.e between 80 and 60 L/h.m^2, than when a transmembrane pressure of 3.5 bar was applied at the beginning. These results are contrary to those obtained previously for wine microfiltration (Ben Amar and Jaffrin, 1989), where average permeate flux increased when transmembrane pressure increased progressively.

Effect of inlet feed velocity

The permeate flux increased with increase in feed velocity (Fig. 5). At about 3.5 bar with a feed velocity of 3 m/sec, the

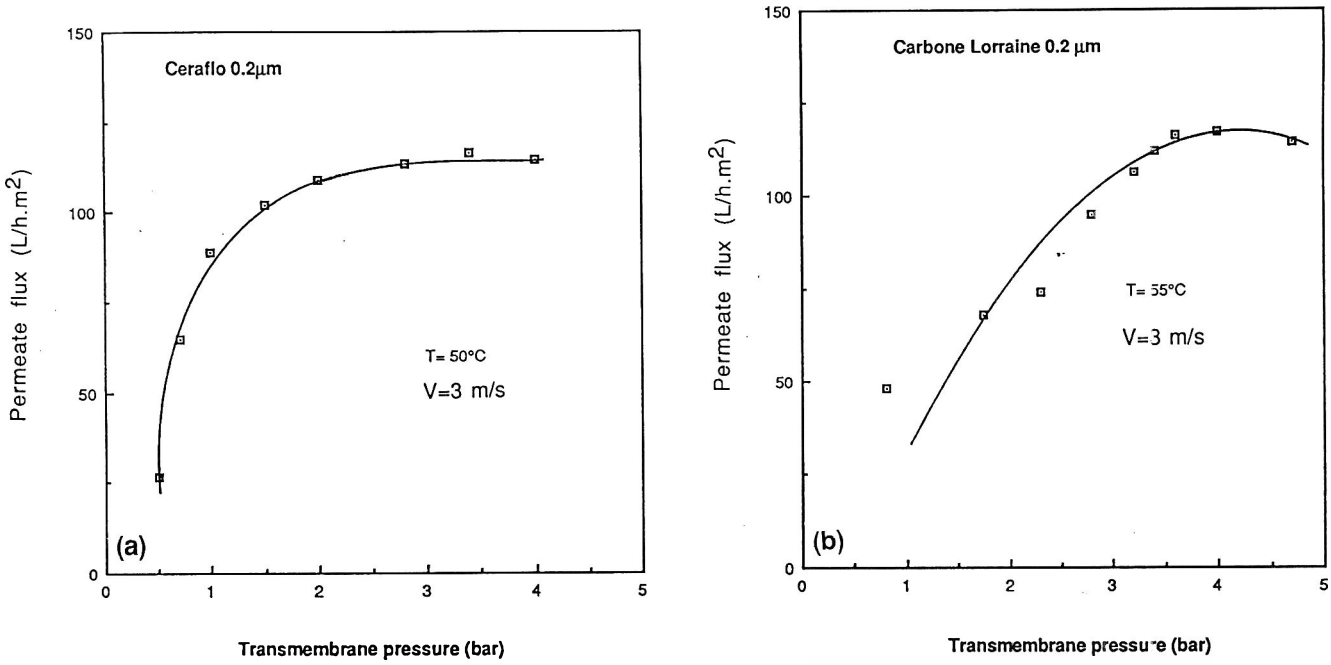

Fig. 3–Effect of transmembrane pressure on permeate flux for apple juice filtration (apple juice treated with pectinase); (b) Effect of transmembrane pressure on permeate flux for apple juice filtration (apple juice treated with pectinase). T = Temperature, V = Inlet velocity.

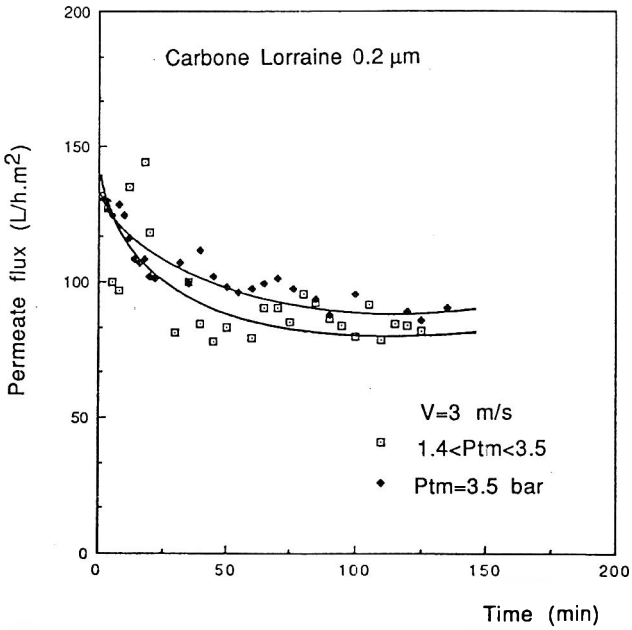

Fig. 4–Effect of transmembrane pressure (Ptm) on permeate flux; □ pressure increased progressively; ◇ optimal pressure applied at the beginning (apple juice without treatment with pectinase); V = inlet velocity.

permeate flux was stable after about 40 min clarification time at 100 L/h.m^2, whereas, at 4.5 m/sec, the permeate flux decreased during the first 80 min and then stabilised at about 150 L/h.m^2.

Effect of temperature

The effect of increase in inlet temperature on permeate flux, with ceramic membranes, is illustrated in Fig. 6. The apple juice, untreated with pectinase, was maintained at 18°C, 30°C and 45°C. The transmembrane pressure was increased up to 2 bar slowly (in about 20 to 30 min), and then the permeate flow rate was measured at different temperatures and times. Permeate flux increased by about 3% for each 1°C increase in temperature. The optimum values of permeate fluxes for apple juice were found at 50 – 55°C (Vrignaud, 1983; Sheu and Wiley, 1987). Higher fluxes at high temperature in comparison with those at low temperature were probably due to reduction in viscosity of the juice.

Effect of backwashing on fouling of membrane

In most cross flow filtration processes the permeate flux declines with time due to membrane fouling. It was therefore advantageous to introduce a procedure which by some fluid mechanical action might partially clean the membrane and restore the initial permeate flux. We investigated backwashing with permeate to remove particles deposited in pores and also on the surface. The backwash pressure (P_c) was from compressed nitrogen and was 2 to 3 bar higher than the internal pressure in the membrane tubes. Backwashing was 2 to 5 sec. The period between backwashing was 2 to 5 min. Variations of flux with time through Ceraflo membranes for juice treated and untreated with pectinase, with and without backwashing are shown in Fig. 7 and 8. The inlet feed velocity and transmembrane pressure were kept constant at 2.77 m/s and 3 to 3.5 bar. We observed that the permeate flux increased with backwashing for both types of apple juice when compared with permeate flux without backwashing. After prolonged operation, the permeate flow dropped slightly in spite of regular backwashing, perhaps because pores became clogged with fine particles which could not be dislodged after backwashing. The permeate flux of depectinized apple juice increased more than the flux of untreated apple juice (Fig. 8), the average value was about 200 L/h.m^2.

The effect of backwashing on permeate flux using Carbone Lorraine membrane is shown in Fig. 9. Surprisingly, in this case there was no appreciable increase in permeate flux with backwashing. This may have been because the Carbone Lorraine membrane is a single tube with very thin walls (1mm), compared to Ceraflo membrane (2mm). The fouling in the

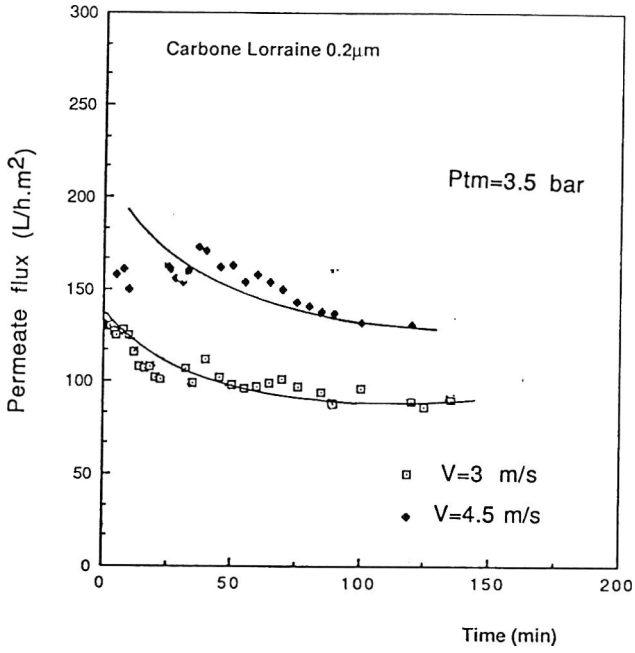

Fig. 5—Effect of inlet feed velocity on permeate flux (apple juice without treatment with pectinase); Ptm = Transmembrane pressure; V = Inlet velocity.

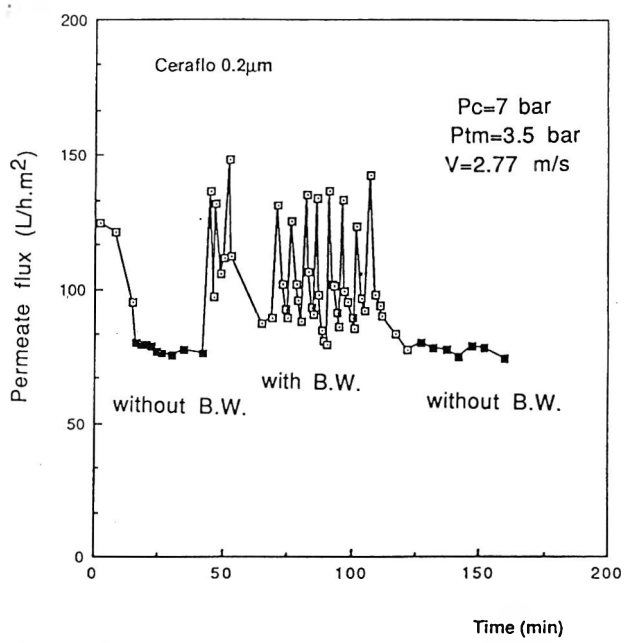

Fig. 7—Effect of backwashing (B.W.) on fouling of membrane (apple juice without treatment with pectinase); Ptm = Transmembrane pressure; V = Inlet velocity; Pc = Backwash pressure.

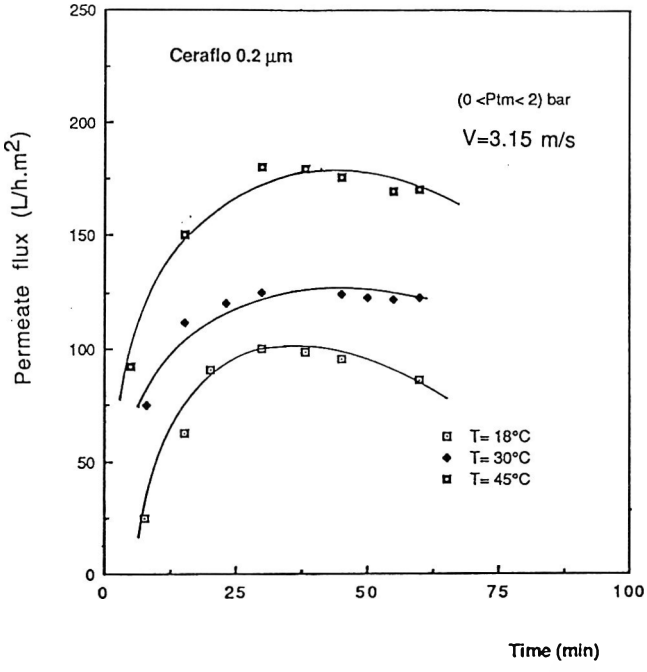

Fig. 6—Effect of temperature (T) of apple juice on permeate flux (no pectinase); Ptm = Transmembrane pressure; V = inlet velocity.

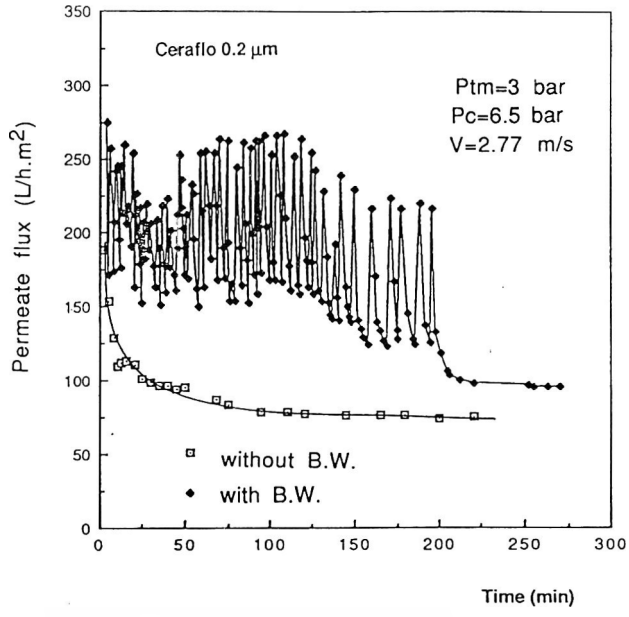

Fig. 8—Effect of backwashing (B.W.) on fouling of membrane (apple juice treated with pectinase); Ptm = Transmembrane pressure; V = Inlet velocity; Pc = Backwash pressure.

pores may have been less and thus backwashing had practically no effect.

Effect of pulsating entry flow on fouling of membrane

The system of imposing flow and pressure wave on entry flow (Fig. 2) was tested for controlling membrane fouling, by increasing mass transfer and reducing formation of the concentration polarization layer near the membrane surface. By producing bursts of accelerated velocity, pulsations may also reduce membrane fouling. This technique has been found suitable for plasma separation (Jaffrin et al., 1986), wine clarification (Ben Amar et al., 1987) and milk ultrafiltration (Bouzaza, 1989) and produced about 50 to 70% increase in permeate flux. The variation of flux for steady flow and pulsating flow with Ceraflo and Carbone Lorraine membranes is shown in Fig. 10 and 11. The frequency (f) and displaced volume (ΔV) in both cases were 1.33 Hz and 84 cm^3, respectively. These values were optimal for the inlet velocity and transmembrane pressure and were obtained from experiments conducted at various frequencies and displaced volumes (Ben Amar et al., 1987). Using pulsations, permeate flux increased from 200 to 250 L/h.m^2, for 200 min, with Ceraflo membranes and from 175 to 225 L/h.m^2, for about 100 min, with Carbone Lorraine membranes. That was about 25–30% immediate increase in permeate flux. We observed permeate flux decreased slowly with time under

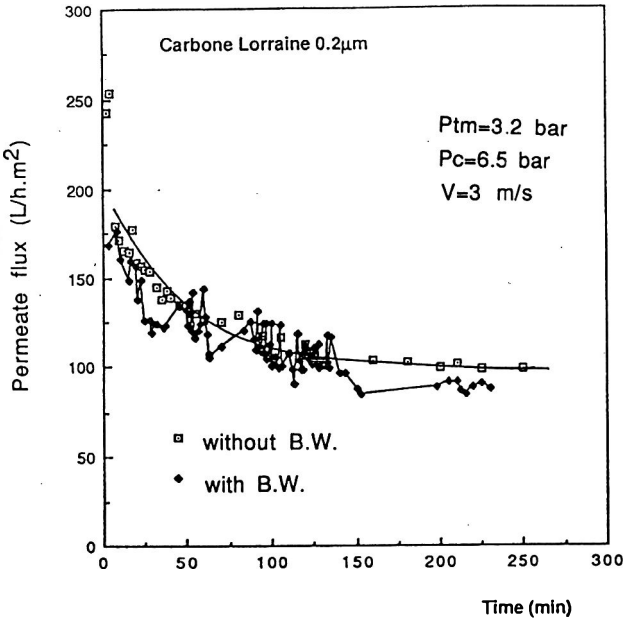

Fig. 9—Effect of backwashing (B.W.) on fouling of membrane (apple juice treated with pectinase); Ptm = Transmembrane pressure; V = inlet velocity; Pc = Backwash pressure.

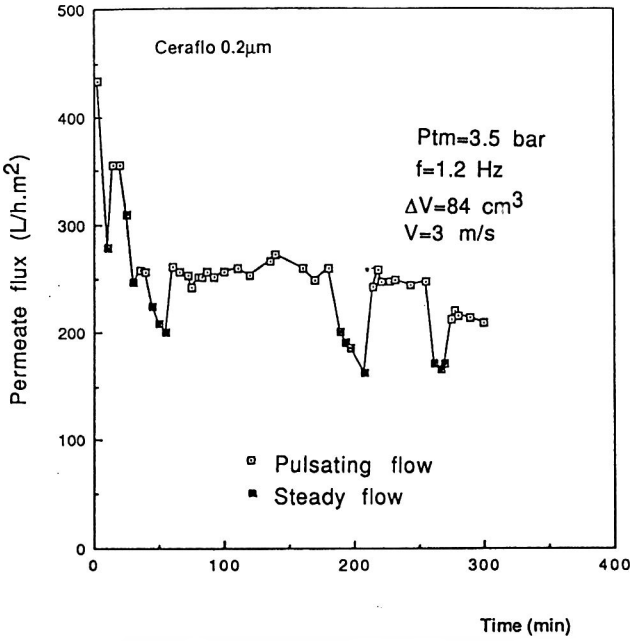

Fig. 10—Effect of pulsating inlet flow on fouling of membrane (apple juice treated with pectinase); Ptm = Transmembrane pressure; f = Frequency of pulsations; ΔV = Displaced volume; V = inlet velocity.

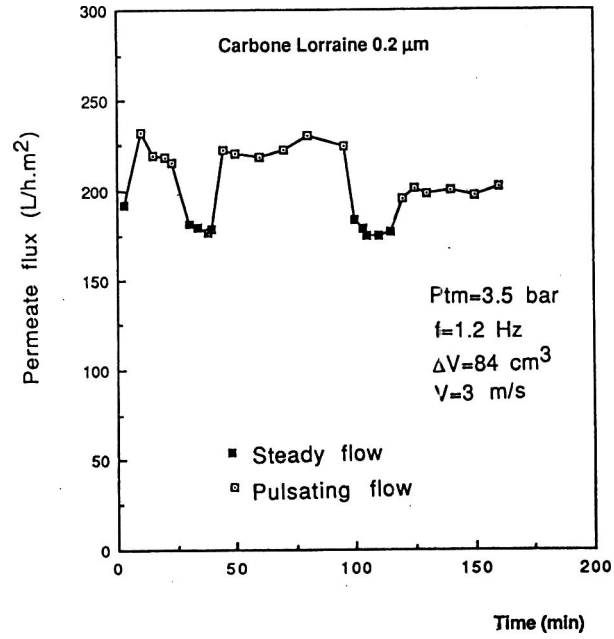

Fig. 11—Effect of pulsating inlet flow on fouling of membrane (apple juice treated with pectinase); Ptm = Transmembrane pressure; f = Frequency of pulsations; ΔV = Displaced volume; V = Inlet velocity.

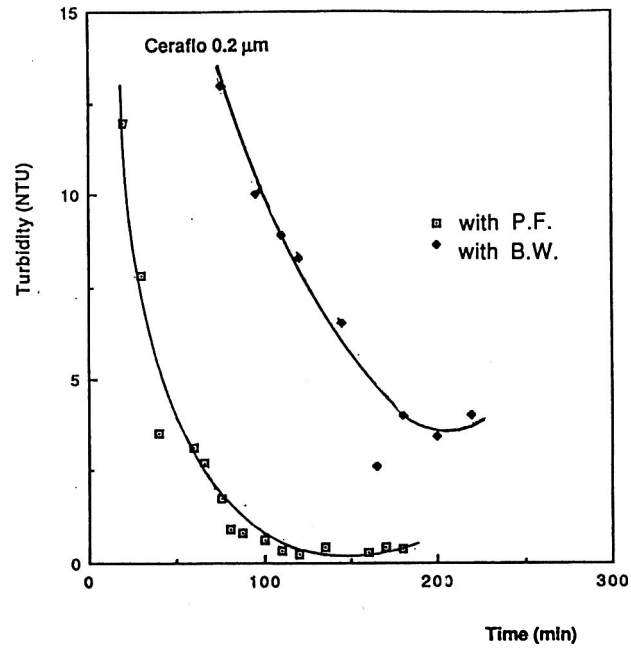

Fig. 12—Effect of time on turbidity of depectinized apple juice using pulsating inlet flow (P.F.) and backwashing (B.W.).

either condition, but permeate flux remained higher with pulsating flow than with steady flow rate. We noted an increase of more than 100% (Fig. 10) on average, compared with flux from steady flow during the entire process (Fig. 8). This showed fouling was partially inhibited by pulsations during the first period of filtration. This may indicate pulsations reduce formation of a fouling layer of rejected particles on the membrane by exerting a "scouring" effect.

Turbidity

The turbidity of the raw juice before filtration was higher than 200 NTU. The turbidity of the permeate with pulsating flow dropped from 15 NTU after 10 min filtration to 0.4 NTU after 120 min and remained stable (Fig. 12). With backwashing the permeate turbidity remained consistently higher, dropping to about 3 NTU after 3 hr.

CONCLUSIONS

RAW APPLE JUICE was successfully clarified by cross flow microfiltration using ceramic or carbon membranes. Optimum operating parameters were 50–55°C, transmembrane pressure 3–3.5 bar and inlet flow velocity 4–4.5 m/sec. Pectinase treatment of apple juice increased permeate flux. With ceramic membranes fouling was controlled by periodical backwashing

and increased permeate flux was maintained up to 3 hr. However permeate turbidity was increased as if the short bursts of retrofiltration dislodged particles from the membranes which reentered the permeate. Another possibility was that low turbidity in the normal process was due to sieving action of a layer of deposited particles on the membrane rather than the membrane itself. By greatly disturbing this layer at regular intervals, backwashing may have reduced its sieving action and therefore increased turbidity. The superimposition of pulsations on inlet flow increased the permeate flux by 70%, up to 250 L/h.m^2 for both ceramic and carbon membranes. At the same time the turbidity remained level at 0.4 NTU (after 2 hr filtration) presumably because pulsations had less disturbing action on the layer of deposited particles than backwashing. The thickness of this layer may have been reduced due to increase in mass transfer by pulsations. This would have increased the permeate flux but the layer would not be destroyed as by backwashing and would have retained its sieving action.

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A Test for Evaluation of the Serum Separation Potential of Tomato Ketchup

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ABSTRACT

The tendency of tomato ketchup to separate into a structural solids phase and a serum phase was measured by placing a small amount of ketchup on a wire screen mounted at the bottom of a plexiglass tube. The screen retained structural solids along with "bound" serum while "unbound" serum drained through and collected in a holder tube below the screen. Rate and degree of separation were particularly applicable to study separation potential of single serving ketchup pouches. Although reducing storage temperature decreased initial rate of serum separation, final amount of serum loss was independent of temperature.

INTRODUCTION

AMONG THE PROPERTIES of ketchup that determine its grade is the tendency of the product to retain the liquid portion in suspension (Anon, 1953). Phase separation in tomato ketchup is particularly apparent in some individual serving pouches but tests provide little information concerning serum separation potential of ketchup. Factors determining the quality of tomato products in terms of consistency have been identified. However, "thin" or "thick" ketchups may have either a high or low degree of serum separation (Davis et al., 1954; Twigg, 1959; Marsh et al., 1979b; Shomer et al., 1983). In the blotter test a ketchup sample is applied to a filter paper and the distance of serum flow on the paper, measured after an appropriate time, is used as an indicator of separation potential (Nelson et al., 1957). Twigg (1959), comparing the blotter test with the rate of filtration test (filtration under vacuum of diluted sample, McCollah et al., 1950) selected the blotter as more appropriate for serum separation measurements. However, Palma (1983) showed the results obtained using the blotter test were dependent on the rate of evaporation of water from the filter paper, that is, upon the humidity surrounding the blotter. Also, he found no relationship between serum flow on the blotter and separation of ketchup in individual serving pouches. Another serum separation indicator is a new type of Bostwick consistometer which measures distance between the point of colorless liquid and the red body in a given period of time (Gould, 1983). Results from a gravity sedimentation test (Robinson et al., 1956; Smit and Nortje, 1958) were also correlated with tendency of serum for separation (Shomer et al., 1983). Serum separation in tomato juice was also measured by Caradec et al. (1985) by monitoring the amount of serum drained through a 60° cone-shaped 42 mesh screen.

However, in single portion ketchup packs, individual pouches can have very different degrees of separation, even though filled from the same, presumably uniform, sub-batch of ketchup. Thus study of only the bulk properties of ketchup is clearly not sufficient to understand serum separation in pouches. The effect of various parameters on serum separation cannot be studied unless reliable quantitative measurements can be obtained. The purpose of this study was to develop a reliable test indicative of the serum separation potential of ketchup in individual serving pouches.

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MATERIALS & METHODS

Ketchup sample preparation

A typical homogenized commercial ketchup (McCormick and Company Inc., Hunt Valley, MD) was used. The effect of storage temperature on serum separation was then studied using nonhomogenized ketchup samples prepared from 32° Brix concentrate tomato paste. The paste was obtained from tomatoes of the UC 204 variety using conventional "Hot Break" or "Cold Break" procedures. Batches of 200–1000g ketchup were prepared using the method described by Marsh et al. (1979a). Details of the procedure, starting from raw tomatoes and ending with ketchup, are reported elsewhere (Stoforos, 1984).

Serum separation test

For serum separation measurements, a "screen tube" was constructed by the Food Science and Technology Department shop. A stainless steel screen (60 mesh/inch, wire size 0.190 mm, openings 0.234 mm) was mounted at one end of a 2.54 cm outside diameter plexiglass tube as shown in Fig. 1. A 50 mL polypropylene centrifuge tube (Nalgene #3110-9500) was used to hold the screen tube.

At the beginning of each test, the weights of holder tube, and the screen tube and holder tube combined were measured (±0.1 mg). The combined tubes were placed into a test tube rack and, using a plastic pipet, 5-5.5 g ketchup samples were carefully applied on the screen. Care was taken to not force the sample through the screen and to ensure that the sample was forming an even, slightly domed shape on the screen without extending up the sidewalls of the screen tube.

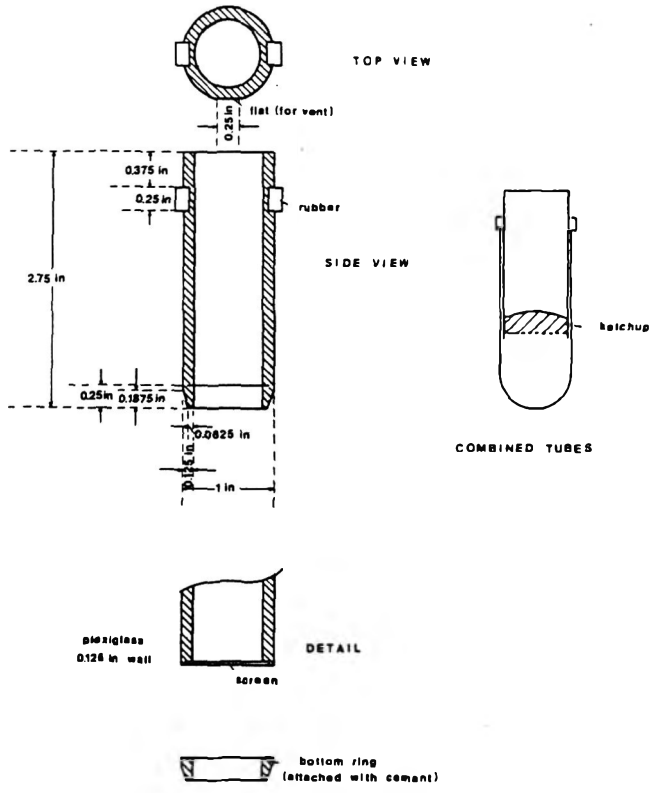

Fig. 1—Schematic diagram of the screen tube.

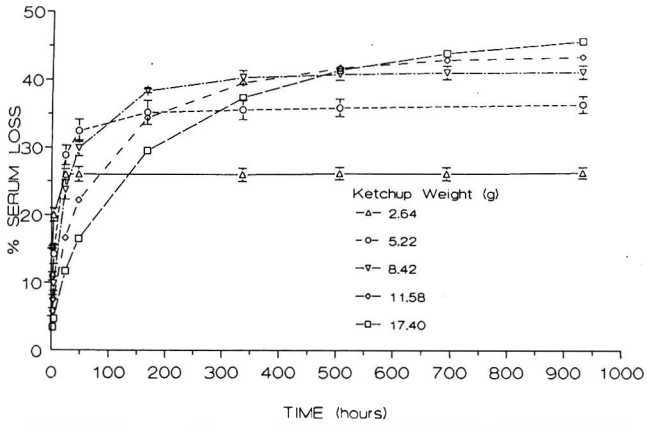

Fig. 2—Effect of ketchup sample weight on serum separation. Each point represents the average of three replicates while each bar indicates one standard deviation.

Table 1—Coefficients associated with Eq. (1) for the experiments with different ketchup sample weight

Ketchup weight (g)[a]	A (hr)	B	R^2
2.64 ± 0.10	0.0600	0.0372	0.989
5.22 ± 0.09	0.1471	0.0282	0.987
8.42 ± 0.09	0.2625	0.0267	0.983
11.58 ± 0.17	0.3416	0.0302	0.957
17.40 ± 0.39	0.6166	0.0344	0.964

[a] Average of three replicates ± one standard deviation

No attempts to change the shape of the sample were made. In case of unevenly delivered sample, the test was redone. The weight of the combined tubes with the ketchup sample was obtained at the beginning and the end of each test in order to determine the sample weight and to ensure that no weight loss occurred during the experiment. To prevent evaporation, the tubes with the ketchup sample were placed in a desiccator containing about 3.5 cm "artificial" serum in the bottom. The artificial serum was prepared using the same method as that for ketchup preparation (Stoforos, 1984), but with high fructose corn sweetener (42° Be) substituted for tomato paste to result in the same soluble solids content as the ketchup samples. Sodium benzoate (0.2% based on the added water) was added as a preservative.

At intervals, the combined tubes were removed from the desiccator, the condensed moisture on the exterior of the holder tube was wiped off, the screen tube was carefully separated from the holder tube, and the weight of the holder tube with accumulated serum was measured. After each weighing, the screen tube was replaced in its holder and returned to the desiccator. Weighing intervals of 1, 2, 4, 24, and 48 hr and 1, 2, 4 wk provided sufficient data to generate a smooth curve for the ketchup samples studied. Other ketchup formulations may require variations of this schedule. The test was considered complete when two consecutive measurements were the same. The percent serum loss (SL) was calculated from

$$SL = \frac{\text{Serum weight}}{\text{Ketchup weight}} \times 100$$

Each test was performed in triplicate.

Ketchup pouches

In order to investigate the separation of serum as it occurs in the pouches, transparent pouches, of dimensions similar to commercial ones (flat outside dimensions 4 × 7.5 cm), were made. The flat stock material used for the pouches was composed of nonheat-sealable, nitrocellulose-coated, cellophane which was adhesively laminated on one side to 2.0 mil clean, medium density, polyethylene. The pouches were fabricated so that the polyethylene was the heat sealable inner layer, as used by McCormick and Company, Inc. Pouches were cold filled with 9–10g ketchup, allowing for a headspace volume, and the top sealed using a bartype heat-sealer.

Preliminary data indicated pouches filled from the same sub-batch of ketchup had different degrees of serum separation, depending on size and position of the bubble resulting from the air incorporation as headspace during sealing. The orientation of the pouches during storage affected both the air bubble position and the slope of the ketchup surface which was in contact with the air bubble. Serum separation was affected by these factors.

Gravity sedimentation test

A gravity sedimentation test was also performed and correlation between this test and the serum separation potential was tested. During the gravity sedimentation test, one part ketchup was diluted with six parts (by weight) water or the artificial serum previously described. The samples were allowed to settle at least 1 month in 18 × 50 mm rimless culture tubes covered with parafilm. During this time, the P/L ratio versus time was calculated by measuring the solids precipiate height (P) and the total height (L) of the sample. The 1:7 dilution was used to obtain accelerated separation, since at lower dilutions many homogenized ketchup samples show little separation in the time scale of the experiment.

RESULTS & DISCUSSION

Determining optimum sample mass

Preliminary experiments were performed to determine the preferred sample mass to be used during serum separation tests. Different weights of homogenized commercial ketchup samples were tested. The results of percent serum loss versus time are presented in Fig. 2. The asymptotic trend of the percent serum loss versus time curves was also observed in all subsequent experiments on serum separation [about 80 different experimental conditions (Stoforos, 1984)]. A regression equation found to adequately predict percent serum loss as a function of time, in all cases, was

$$SL = \frac{t}{A + Bt} \qquad (1)$$

where t represents the elapsed time in hours, and A (hour) and B (dimensionless) are empirical constants. From Eq. (1), taking the limit as time approaches infinity, we found that 1/B represents the final percent serum loss. By differentiating Eq. (1) with respect to time and taking the limit of the resulting expression as time goes to zero, 1/A can be shown to represent the initial rate of serum loss. The coefficients A and B, for the preliminary experiments, are presented in Table 1. As can be seen from Fig. 2 and Table 1, the sample weight affected both the rate and the final amount of serum loss. With a correlation coefficient $R^2 = 0.989$ the following linear relationship was found to describe the inverse of the initial rate of serum loss (coefficient A, Table 1) as a function of the sample weight (SW in grams)

$$A = 0.0370\ SW - 0.0495 \qquad (2)$$

From the above expression, as the sample weight increased, the initial rate of separation (1/A) decreased. This was probably due to the fact that the average distance the serum has to flow before separation was further for larger sample size.

The percent serum loss after 932.5 hours (FSL) correlated with sample weight through the following expression

$$\frac{1}{FSL} = 0.0487\ \frac{1}{SW} + 0.0189 \qquad (3)$$

with a correlation coefficient $R^2 = 0.988$.

Equation (3) indicates that as sample weight increased, the amount of serum loss (at 932.5 hours) increased, approaching a limiting value of 53% for the particular ketchup sample studied. The increase of serum loss with increasing sample weight may be due to the fact that a certain amount of serum, dependent only on the cross sectional area of the surface through which the serum flows, is held by the porous bed which is created by the structural solids of the ketchup. An additional reason might be the change of sample geometry that has been observed for large samples (>8 grams), from cylindrical to

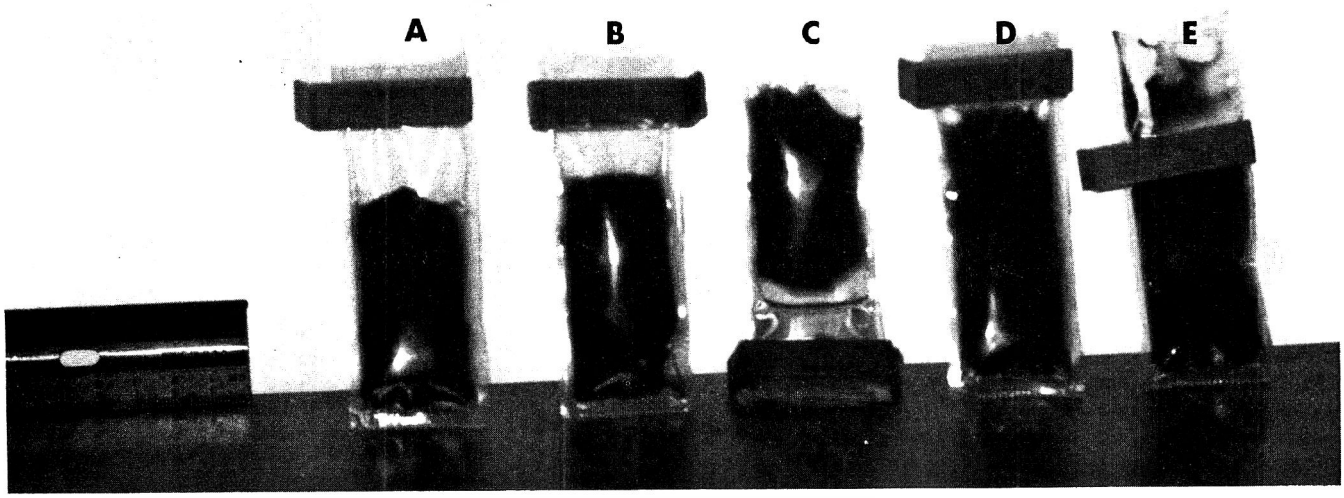

Fig. 3—Effect of air bubble size and position on serum separation of ketchup in pouches.

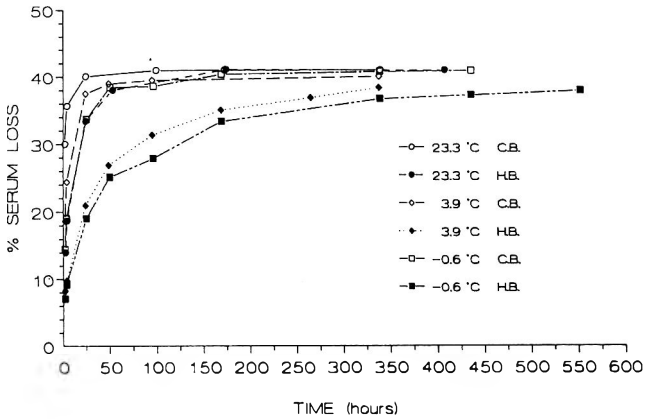

Fig. 4—Effect of temperature on serum separation. (C.B. = Cold Break, H.B. = Hot Break)

Table 2—Coefficients associated with Eq. (1) for the experiments studying the effect of storage temperature

Sample	T (°C)	A (hr)	B	R^2
Cold Break	23.3	0.0164	0.0242	1.000
Cold Break	3.9	0.0623	0.0246	0.998
Cold Break	−0.6	0.0926	0.0249	0.991
Hot Break	23.3	0.1211	0.0245	0.996
Hot Break	3.9	0.2105	0.0314	0.941
Hot Break	−0.6	0.2371	0.0321	0.962

conical. A shrinkage of the ketchup resulted in an increase in the exposed surface of the sample. Note, that this effect was also dependent on the screen tube dimensions.

From the above discussion, it is clear that the sample weight was a factor that should be standardized during the serum separation tests. Wishing to (1) maximize the amount of final serum separation, in order to better distinguish between different ketchup samples, (2) minimize the time required to reach the final separation, and (3) conserve material, the 5-g sample weight was selected as the standard sample weight for the proposed serum separation test.

During development of the test, several experiments were performed. In one, the test was performed outside the desiccator. In another, the screen tube did not have the flat portion for venting (Fig. 1), and in a third, the entire screen tube was placed inside the holder tube and covered with a plastic stopper. In these experiments, a variation of 20 to 50% (expressed as the percent of one standard deviation on the average value of 5 replicates) was found. The final form of the test, as it is described in the Materials and Methods Section gave a variation in the range of 1 to 3% for most subsequent experiments (Stoforos, 1984).

Single serving pouches

The importance of air bubble size and position on serum separation of ketchup in pouches can be seen from Fig. 3. This photograph was taken 1 month after the pouches had been filled with homogenized commercial ketchup samples and stored as shown. In pouch E, where there was no void space, no serum separation was observed. The bottom right side of pouches B and D showed small air bubbles almost filled with serum. From pouches A and B, we could see an analogous effect of slope of the ketchup surface which is in contact with the air bubble. In pouch B, where the ketchup air interface was held in horizontal position, no serum was observed on top. However, there was some serum on the top right side of pouch A where the ketchup was slightly mounded. Pouch C illustrated the role of the air bubble as a serum-holding shell.

It can be inferred, from Fig. 3, that natural serum separation for ketchup in pouches was due to drainage of the serum through the solids matrix rather than to the settling of insoluble solids. We could also conclude that the developed test simulated this phenomenon. The function of the screen used in our test was only to support the ketchup sample. The "filtration" of the serum was done through the insoluble solids of the ketchup. The chosen screen size was the one with maximum openings able to hold every ketchup sample studied (Stoforos, 1984). The minimum size of perforations through which the whole sample would start to flow was not the same for all the samples. There were samples that did not flow through 4 mm^2 perforations. However, significant differences were observed between various samples (for example, comparing homogenized with nonhomogenized ketchups). Those results will be reported later.

Effect of storage temperature

In ketchup pouches, we observed that lowering storage temperature slowed serum separation. To examine the effect of temperature on serum separation, the separation test was performed at three different temperatures, (23.3, 3.9, and −0.6°), on hot break and cold break ketchup samples. The results are presented in Fig. 4.. The corresponding coefficients associated with Eq. (1) are presented in Table 2. For both hot and cold

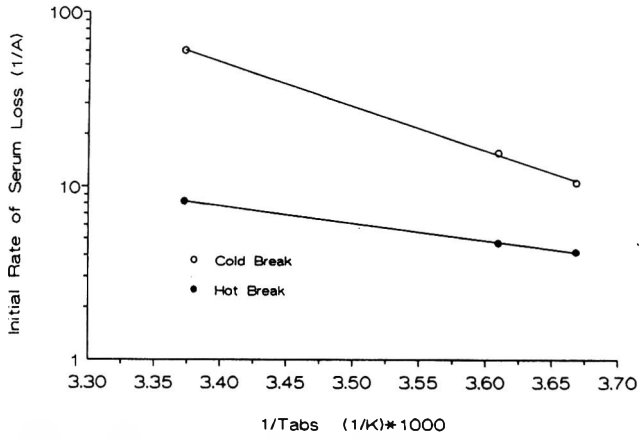

Fig. 5—Arrhenius plot showing effect of temperature on initial rate of serum separation.

break samples, the initial rate of serum loss (1/A, Table 2) followed the Arrhenius Equation (Fig. 5).

With a correlation coefficient $R^2 = 1.000$, the results for the hot break samples were described by

$$1/A = 1.86 \times 10^4 \exp(-2.29 \times 10^3/T_{abs}) \quad (4)$$

for T_{abs} being the absolute temperature (K) and 1/A in hour^{-1}.

For the cold break sample, the Arrhenius expression with an $R^2 = 0.999$ was

$$1/A = 1.93 \times 10^{10} \exp(-5.80 \times 10^3/T_{abs}) \quad (5)$$

Though the initial rate of serum separation decreased as temperature decreased, the final amount of serum loss was almost independent of storage temperature.

Gravity sedimentation test

No correlation was found between results using the gravity sedimentation test (performed on samples diluted with water or artificial serum) and either the serum separation test or the serum separation as it occurred in pouches of ketchup (Stoforos, 1984). The gravity sedimentation test better simulated the serum separation of ketchup in bottles, which is probably due to the settling of the solids matrix, and results in a layer of serum above the product. This confirms that serum separation in pouches is due to liquid drainage, and that the serum separation test is a more appropriate test.

SUMMARY

A TEST simulating serum separation of tomato ketchup in individual serving pouches was developed. The ketchup sample was supported by a screen, and the serum drained through the insoluble solids matrix in a mechanism similar to that occurring in natural separation in pouches. In contrast to previously used tests, this test quantified both rate and final amount of serum loss. A saturation-like model correlated serum loss with time, eliminating the need for prolonged experiments.

By means of the test, the effect of storage temperature on serum separation was studied. Although reducing storage temperature decreased initial rate of separation, the final amount of serum loss was independent of temperature.

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Effect of Manufacturing Conditions on Rheology of Banana Gelified Milk: Optimization of the Technology

AMEL HAMZA-CHAFFAI

ABSTRACT

Banana Gelified Milk (BGM) was processed under four operating conditions: cooking time (15–85 min), cooking temperature (65–95°C), starch concentration (7–11 $g.L^{-1}$), and carrageenan concentration (2–6 $g.L^{-1}$). Rheological measurements were obtained using a concentric cylinder viscosimeter with shear rate ranging from 5 to 80 sec^{-1}. The consistency coefficient, the flow behavior index, the apparent viscosity and the yield stress were determined and compared to those of BGM prepared by an industrial process. An experimental design with 2^4 experiments was used to optimize BGM preparation. Proposed mathematical models showed that the apparent viscosity was mainly affected by carrageenan concentration. However, cooking temperature had greatest effect on yield stress. An optimized product was prepared reducing cooking time and temperature. Carrageenan was optimized to 3.5 $g.L^{-1}$. This provided a product with good texture and acceptable taste. This was confirmed by sensory evaluation.

INTRODUCTION

RECENT PROGRESS in scientific and technological research, coupled with the recognized nutritive value of milk, have led to a new concept of milk as a readily processed raw material for development of dairy food. Webb et al. (1983) showed that traditional and new dairy food products differ widely in chemical and physical characteristics.

Considerable importance is given to certain physical properties of milk products, such as hardness for butter and viscosity, spreadability, and standing-up qualities for foams. These properties determine the consistency of the product and influence its acceptability to consumers (Awadhwal and Singh., 1985).

Flavoured Gelified Milk (FGM) is widely consumed as a dessert. It is made of pasteurized or sterilized milk, sucrose, flavoring materials (chocolate, vanilla, banana. . .), stabilizers, thickeners, and gelifying substances (Viesseyre, 1979). For the FGM preparation, milk is generally pasteurized, homogenized, then cooled and ingredients are added afterwards. Mixing and cooking are carried out under variable temperatures (60 to 90°C). After cooking, the milk is sterilized and as a final step aroma is incorporated before packaging.

Gelified milk composition varies according to aroma and gellifying agents used, such as starch, carrageenan, alginates, pectins, and agar-agar. They serve as stabilizers, thickeners and structure forming (gelling) agents. These functional properties are related in part to their ability to imbibe and retain a large amount of water and to interact with this water in solution (Wallingford and Labuza, 1983).

BGM is a type of FGM in which the key step of preparation is cooking, where starch and carrageenan interact with milk and other components under the influence of heat. Starch, a polysaccharide isolated from some plants (e.g. corn, rice, etc), swells under the combined effect of water and heat. This treatment leads to amylose and amylopectin depolymerization by random chain spilling (Doublier et al., 1986). Thermal energy allows water to pass through the molecular network but as starch granules swell, their density decreased and they eventually remain in suspension, thus increasing viscosity of the solution (Campbell et al., 1984). Carrageenan, a readily available polysaccharide from seaweed, is nontoxic and widely used as a food additive (Linko and Larinkari, 1980). It reacts with positively charged proteins to form a viscous solution at low concentration. The viscosity is dependent on temperature, pH, concentration, type of carrageenan molecules, and the medium (Kalab, 1979; Wallingford and Labuza, 1983). The formation of carrageenan gels occurs due to double helical junction by cooling a hot solution (Sugget, 1974). With potassium ions and amino-acid derivatives, which are present in milk, carrageenan may form a gel similar to agar-agar gel (Cheftel et al., 1978).

As with any other milk product, the rheological properties of BGM are affected by its composition, manufacturing conditions, and the ability of gellifying agents to produce a continuous three-dimensional gel network.

Processing variables affecting flow behavior and textural properties of BGM include cooking time, temperature and gelifying concentration (Holt et al., 1984). BGM, similar to milk desserts, yogurt, and cream, may be described as a non-Newtonian food material, which shows a dependence of apparent viscosity on shear rate.

One of the most generalized mathematical models, containing a yield stress term, used for these materials is the "Hershel-Bulkley" model (1):

$$\tau = \tau_o + K\gamma^n \qquad (1)$$

τ represents the shear stress (Pa), τ_o the yield stress (Pa), K the consistency coefficient ($Pa.s^n$), n the flow behavior index and γ the shear rate (sec^{-1}).

This model was used to determine some rheological and textural properties of BGM (Heldman, 1975; King, 1980). The flow behavior index "n" is less than 1 for shear thinning (pseudoplastic) fluids, greater than 1 for shear thickening (dilatant) fluids, and equal to 1 for Newtonian fluids, where the consistency "K" is equal to the viscosity (Rao et al., 1986).

The purpose of this investigation was to characterize rheological changes in BGM and study the effect of processing conditions. Three technological parameters were varied: (1) cooking time, (2) cooking temperature, (3) starch concentration, and (4) carrageenan concentation at constant cooking temperature and duration. The combined effect of these parameters was shown by establishing mathematical models. These models were used to optimize BGM preparation.

MATERIALS & METHODS

Raw materials and processing

Raw, whole milk powder (26% fat), carrageenan, sugar (sucrose), banana, aroma, and starch were kindly supplied by S.T.I.L. (Société Tunisienne des Industries Laitières, Tunis). The reference product was prepared according to the industrial process: 125g powder were dissolved in 900 mL warm water, then stored at 4°C 12 hr. Pasteurization was carried out at 83°C for 30 sec in a plate heat exchanger. 75g sucrose, 10g starch, and 4g carrageenan were mixed at 30°C, then cooked according to selected conditions, and the product sterilized at 140°C for 3 sec. Before packaging, banana aroma was added. BGM was prepared by different cooking methods as shown in results (Table 1).

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Table 1—Power law constants and R^2 for different cooking conditions (variation of one parameter)

Parameter	ζ_o (Pa)	n	K (Pa.s^n)	η (mPa.s)	R^2 Casson	R^2 Power law
Time (min)[a]						
15	19.301	0.668	2.007	473.38	0.939	0.987
30	22.298	0.600	2.707	473.51	0.995	0.996
45	22.048	0.635	2.722	484.53	0.964	0.987
60[e]	23.282	0.600	2.970	519.91	0.996	0.997
75	19.991	0.618	2.673	506.99	0.990	0.917
Temperature (°C)[b]						
65	11.508	0.566	2.924	442.087	0.971	0.963
70	16.441	0.703	1.669	458.55	0.904	0.920
75	22.948	0.578	2.584	460.879	0.999	1.000
85[e]	23.282	0.600	2.970	519.911	0.996	0.997
95	28.427	0.621	2.880	553.618	0.993	0.995
Starch (g.L^{-1})[c]						
7	19.839	0.587	2.412	399.472	0.998	0.999
8	19.062	0.669	2.830	670.757	0.963	0.968
9	24.967	0.674	2.188	529.586	0.922	0.932
10[e]	23.282	0.600	2.970	519.911	0.996	0.997
11	21.524	0.683	2.186	550.471	0.924	0.934
Carrageenan[d]						
2	11.120	0.580	2.097	336.676	0.996	0.990
3	21.944	0.558	2.782	405.74	0.926	0.999
4[e]	23.282	0.600	2.970	519.912	0.996	0.997
5	29.155	0.635	3.029	617.302	0.968	0.971
6	32.431	0.598	4.000	695	0.996	0.997

[a] 85°C, 10 g.L^{-1} Starch, and 4 g.L^{-1} carrageenan
[b] 60 min, 10 g.L^{-1} starch and 4 g.L^{-1} carrageenan
[c] 85°C, 60 min and 4 g.L^{-1} carrageenan
[d] 85°C, 60 min and 10 g.L^{-1} starch
[e] Reference product

Viscosity and rheological measurements

All viscosity and rheological measurements were performed on one or two day-old gellified milk with a concentric cylinder viscosimeter (CONTRAVES, STV) over a common range of shear rate up to 5 sec^{-1} connected to an asynchronous motor rotating at five different speeds.

All readings were made at 18°C. A refrigerated circulating water bath was used to keep temperature constant. The shear-rates (γ) were calculated using speed values (ω) and the spindle dimensions (r,h) (Launay, 1981). The 'C' spindle used had a 10 mm radius 'r' and 45 mm length 'h'. The cylinder was filled with 18–22 mL of product. Great care was taken to avoid air pockets.

Data analysis and calculations

The "Hershel-Bulkley" model mentioned above (1) for non-Newtonian fluids was used. The yield stress τ_o K and n were estimated using the least squares method from square root and double logarithmic plots respectively. The estimation of all rheological parameters is discussed below:

Yield stress was obtained using Casson equation (2):

$$\sqrt{\tau} = \sqrt{\tau_o} + K_c\sqrt{\gamma} \qquad (2)$$

Performing the regression $\sqrt{\tau}$ versus $\sqrt{\gamma}$ gave $\sqrt{\tau_o}$ as an intercept. Logarithmic transformation of Eq. (1) yielded Eq. (3):

$$\text{Log}\,(\tau - \tau_o)\ \text{Log K} + n\text{Log}\gamma \qquad (3)$$

Linear regression gave Log K as an intercept and n as a slope. All regressions were performed using TSP (time series processors) (Version 4.0).

The apparent viscosity defined as the ratio of shear stress to actual shear rate was calculated using Eq. (5) deduced from Eq. (4) for $\gamma = 77.92$ sec^{-1}:

$$\tau = K\gamma^n \qquad \eta = \tau/\gamma \qquad (4)$$

$$\eta = K\gamma^{n-1} \qquad (5)$$

Optimization using an experimental design

The rheological characteristics, i.e. the apparent viscosity (η), the consistency coefficient (K), the flow behavior index (n), and the yield stress (τ_o) of BGM were related with selected parameters (temperature, cooking time, starch concentration, and carrageenan concentration).

An experimental design with four variables was used. A number of $2^4 = 16$ experiments, consisting in all possible combinations, was carried out. The general model used was:

$$Y = b_o + \sum_{i=1}^{4} b_i X_i + \sum_{i=1}^{4} b_{ii} X_i^2 + \sum_{ij\ (i \neq j)}^{4} b_{ij} X_i X_j \qquad (6)$$

Where Y = apparent viscosity (η), consistency index (K), flow behavior index (n) and yield stress (τ_o). X_i: coded variable ($X_i = +1$ for maximum value of a variable and $X_i = -1$ for the minimum value). x_1, x_2, x_3, x_4 refers to temperature, time, starch concentration, and carrageenan concentration respectively. The passage from natural variable x_i to coded variable X_i is given by the following equations: X1 = (x1-77.5)/12.5 ; X2 = (x2-37.5)/22.5 ; X3 = (x3-11)/3 ; X4 = x4-3.

The model coefficients (b_o, b_{ii}, b_{ij}) were estimated using RATS (regression analysis and time series^2, version 1987). These coefficients were tested by Student test and the final model was chosen using Fisher test (Chatterjee and Price., 1983).

Sensorial evaluation

A group of 15 panelists was selected using a triangle test. Three samples were presented: two samples were identical and each panelists was asked to identify the different sample. Then a series of four optimized BGM together with the industrial product was presented to panelists who were asked to identify each BGM by ranking suitable characteristics such as taste, texture. . . . .Scores were given to each product. An analysis of variance (Hamza-Chaffai, 1990) was used to evaluate differences between the optimized and commercial BGM for significance.

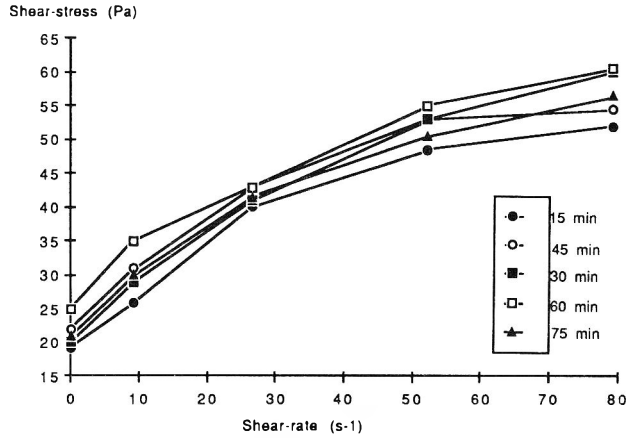

Fig. 1—Flow curves for different cooking times at constant temperature (85°C).

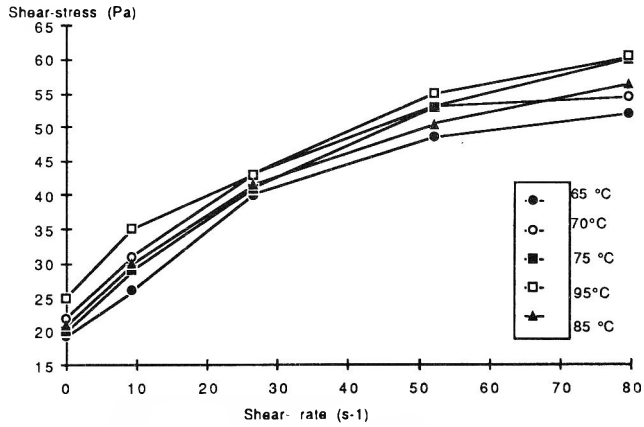

Fig. 2—Flow curves for different cooking temperatures at constant time (60 min).

Table 2—Treatment combinations, rheological data[a] and R^2 for Banana Gelified Milk (Experimental design)

	Parameter				Rheological parameter				R^2 %	
	×1	×2	×3	×4						
Treatment combination	T° (°C)	Time (min)	Starch (g/L)	Carr ($g.L^{-1}$)	η (mPa.s)	K ($Pa.s^n$)	n	τ_o (Pa)	Casson	Power Law
1	65	15	8	2	216.69	0.88	0.68	9.50	0.946	0.952
2	90	15	8	2	277.08	1.87	0.56	14.41	0.995	0.992
3	65	60	8	2	159.43	0.45	0.76	12.49	0.787	0.841
4	90	60	8	2	220.94	1.35	0.58	16.16	0.989	0.993
5	65	15	14	2	256.83	1.26	0.63	9.73	0.990	0.992
6	90	15	14	2	426.32	2.48	0.60	21.22	0.995	0.996
7	65	60	14	2	186.37	0.64	0.72	9.11	0.883	0.904
8	90	60	14	2	407.33	1.68	0.67	16.44	1.000	1.000
9	65	15	8	4	398.61	2.06	0.56	12.68	0.982	0.964
10	90	15	8	4	424.25	2.65	0.63	21.09	0.987	0.974
11	65	60	8	4	528.87	2.02	0.69	5.84	0.975	0.968
12	90	60	8	4	478.59	2.33	0.64	19.17	0.974	0.977
13	65	15	14	4	414.76	1.40	0.72	10.95	0.909	0.925
14	90	15	14	4	342.47	1.98	0.76	32.63	0.766	0.828
15	65	60	14	4	486.91	2.05	0.67	13.72	0.999	0.984
16	90	60	14	4	526.53	3.08	0.54	29.24	0.965	0.968

[a] Mean value for three replications

Table 3—Regression coefficients for the viscosity (η), the consistency index (K), the flow behavior index (n), and the yield stress (τ_o) models

Parameters	Viscosity[a] (η)	K[b]	n[c]	τ_o[a]
Linear				
Constant	358.374	1.737	0.065	15.899
Temperature: X_1	29.566 (t = 2.35)	0.315 (t = 2.909)	−0.028 (t = −2.282)	5.396 (t = 8.801)
Time: X_2	–	–	–	–
Starch: X_3	–	–	–	1.981 (t = 3.231)
Carrageenan: X_4	89.500 (t = 7.1)	0.409 (t = 3.78)	0.0233 (t = 1.896)	2.266 (t = 3.697)
Interactions				
X_1X_2	–	–	–	–
X_1X_3	–	–	–	1.606 (t = 2.61)
X_1X_4	−35.478 (t = −2.74)	−0.203 (t = −1.87)	–	1.971 (t = 3.215)
X_2X_3	–	0.203 (t = 1.87)	−0.217 (t = −1.766)	–
X_2X_4	39.103 (t = 3.10)	0.206 (t = 1.901)	–	–
X_3X_4	−30.022 (t = −2.38)	0.283 (t = −2.61)	–	1.490 (t = 2.43)
$\bar{R}^2$ (Adjusted)	0.887	0.817	0.763	0.933

[a] $p < 0.05$ (significant)
[b] $p < 0.1$ (significant)
[c] $p < 0.2$ (significant)

RESULTS & DISCUSSION

Rheological behavior of BGM

BGM prepared by industrial process was used as a reference. This product was designed to have average composition: 42.52% dry matter, 25.5% fat, 6.5% proteins, 0.15% acidity (lactic acid), 10.36% carbohydrates, and an energy value of 294.5 Kcal per 100g.

The first step in characterizing rheology of BGM was to examine shear stress vs shear rate. Flow curves and flow behavior index n = 0.599 (n < 1) showed that BGM exhibited a pseudoplastic or shear thinning behavior with yield stress τ_o = 23.284 Pa (Table 1). Hershel-Bulkley model (1) was employed to fit viscosimetric data. Linear regression analysis was performed on obtained (τ, γ) data, resulting in value of τ_o from casson equation (2) (R^2 = 0.995). K and n were estimated using Eq. (1) with R^2 = 0.997. The apparent viscosity η = 519.91 mPa.s.

Power law constants were related to the flow and texture of the food product. In fact, the consistency index K increased with increasing solids content (Chhinnan et al., 1985). The flow behavior index (n) was used to measure departure from Newtonian flow (Parnell-Clunies et al., 1986). The yield stress of the fluid related to the force required to initiate the flow or to the stress that was required to move one fluid layer from another. Some investigators have not recognized yield stress as an exact physical parameter, but as a parameter depending on the manner in which the material was handled.

Effect of parameters

Cooking time. To evaluate the effect of cooking time on rheological characteristics of BGM temperature, starch concentration and carrageenan concentration were fixed. Shear stress-shear rate curves are shown in Fig. 1 for different times of cooking. For the range of shear we used, the general power law model (1) described accurately the flow behavior at each time. The highest stress readings were registered for BGM cooked 60 min, followed by t = 45, 30, 75 and 15 minutes, respectively. Also the highest yield stress was observed for t = 60 min, However the lowest shear stresses were shown for 75, 85 and 15 minutes. A possible explanation is that 15 minutes is not long enough to produce gelatinization. On the other hand, a long cooking time (75 and 85 min) caused destruction and retrogradation of the formed structure.

The consistency coefficient (K) and the flow behavior index (n) for each flow curve are given in Table 1. There was an increase in the consistency index with the increasing cooking time until 60 min. The determination coefficients R^2 ranged from 0.98 to 0.99, which indicates that the power law model (1) described well the shear rate-shear stress data. BGM cooked 75 min or more, had significantly reduced apparent viscosity, consistency index and yield stress. This would be of little practical interest because of the destruction of BGM structure and the high cost. The loss of consistency or viscosity was most likely due to failure of the macromolecular polymeric substances (Labuza, 1977) and the following break down of structure. This result is confirmed by the fact that some hydrocolloïds are hydrolyzed by heating a long time.

Cooking temperature. Flow curves of BGM cooked at different temperatures (Fig. 2) showed that all BGM were pseudoplastic with differences in yield stress. The highest shears were observed for high temperatures (75°, 85° and 95°C), with slight differences in yield stress values. At lower temperatures, shear stress and the yield stress decreased. For each cooking temperature yield stress values were substracted from shear stress readings and plotted against shear rate to determine power law constants. Apparent viscosity, power law constants, yield stress, and R^2 for Casson equation and Log - Log plots are given in Table 1. Flow behavior index 'n' was not significantly affected by different cooking temperatures, with most values ranging between 0.56 and 0.70. Cooking temperature effects on apparent viscosity 'η' were evident and followed the same trend as yield stress.

From the above data, we suggest that at low temperatures (T < 65°C) the energy to the system was not enough to affect the crystalline zones of high stability, although this energy was sufficient to increase disorder of the amorphous region. Thus, complete gelatinization was not achieved. At temperatures above 75°C, the additional energy to the system increased mobility of chains in the amorphous regions such that complete gelatinization was achieved (Ravisani et al., 1985).

Ingredient concentration. The apparent viscosity and the yield stress were affected by starch concentration. However, consistency index 'K' and flow behavior index 'n' did not show any apparent trend (Table 1). Increasing starch concentration did not resist in maximum values of viscosity and consistency, because more time and higher temperatures would be required for starch gelatinization. Increases in carrageenan concentration increased the consistency coefficient, yield stress, and apparent viscosity. The flow behavior index ranging from 0.55 to 0.63 indicated that BGM conserved its pseudoplasticity.

Experimental design and optimization. During industrial preparation the original raw materials were subjected to the combined effects of time and temperature. Starch and carrageenan interacted with other components in the medium (water, fat, sucrose,..). The final texture and rheology of BGM was not determined by any one of these parameters, but they all interacted and affected flow behavior of the product. An experimental design with 16 experiments provided a systematic procedure for correlating temperature (x_1) time (x_2), starch (x_3) and carrageenan (x_4) concentration with the described rheological parameters of BGM. The treatment combinations and rheological data are presented in Table 2. Each datum is the average of three replications. Maximal values of viscosity and consistency index occurred at treatment combination (16) (90°C, 60 min, 14 $g.L^{-1}$ starch, and 4 $g \cdot L^{-1}$ carrageenan). This was due to use of maximal quantities of starch and carrageenan, in addition to heating at high temperature for a sufficient time. The greatest value of consistency index was accompanied by minimal values of flow behavior index (treatment 16) and vice-versa (treatment 3). These two parameters varied inversely, that is, consistency increased with decreasing flow.

Maximal viscosities ($\eta > 400$ mPa.s) were obtained for treatment combination 6-8-10-11-12-14-15 and 16. All rheological parameters tended to increase by increasing cooking temperatures and carrageenan concentration. Only the flow behavior index showed negative effect with increasing temperature and no significant action with carrageenan concentration. The regression equations developed from the mathematical model (I), regression coefficients and their significance are summarized in Table 3. Not all linear and interaction terms were statistically significant in predicting the rheological response. The apparent viscosity and consistency index were mainly affected by carrageenan concentration followed by cooking temperature. These results confirmed the observation previously reported (Chinnan et al., 1985) concerning the increase of consistency with increasing solids. No significant effect was observed for time and starch concentration (Table 3).

The yield stress value denoted a minimal stress which must be exerted before flow began. This is interpreted as existence of a gel network structure, which must be broken to allow flow. This gel structure was caused by starch and carrageenan interactions with other components in the medium, under the effect of heating.

The derived models may be used in BGM research and development. When formulating a new product Eq. 7 and 8 may be used to provide desired texture.

$$\eta = 385.374 + 29.56X_1 + 89.5X_4 + 39.103X_2X_4 - 30.22X_3X_4 \quad (7)$$

$$\tau_o = 15.899 + 5.396X_1 + 1.981X_3 + 2.266X_4 + 1.606X_1X_3 + 1.9.71X_1X_4 + 1.490X_3X_4 \quad (8)$$

The equations allow predicting viscosities or yield stress from varying concentrations of starch, carrageenan, time and temperature. Many possibilities are offered, and therefore we can select an economic and efficient combination. Trials with reduced carrageenan concentration or with starch substituting for carrageenan are interesting because starch is readily available and inexpensive compared to carrageenan. Considering measured values, apparent viscosity of 412.50 mPa.s and yield stress of 19.324 Pa were obtained using a treatment combination of 80°C, 45min, 10 $g.L^{-1}$ starch, 3.5 $g.L^{-1}$ carrageenan.

Reducing temperature, time, and carrageenan concentration is of economic interest. BGM was prepared using this combination in the laboratory and in the dairy. The rheological measurements on the dairy product were close to those of the laboratory. This was confirmed by sensory studies. The optimized product had a good structure and acceptable taste.

CONCLUSION

THE BGM DEVELOPED by our optimization, using a new combination of technological parameters, had a stable gel structure with no significant modification of rheological characteristics. This system has some economic advantages: (1) reduction of cooking time from 60 min to 45 min leads to better productivity; (2) energy is conserved from reduction of cooking temperature; and (3) the lessening of carrageenan use allows lower cost.

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Lactobacillus plantarum and *Enterobacter Cloacae* Growth In Cucumber Extracts Containing Various Salts

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ABSTRACT

Salt substitutes included chlorides, sulfates and phosphates at 25%, 50% and 100% ionic strength equivalent to a concentration of 1M NaCl. At 100% NaCl substitution, chloride salts provided the most selective environment and sulfates showed no selectivity, whereas phosphates suppressed microbial growth. Substitution of sulfates for NaCl generally increased generation times for both microorganisms. Substitution of KCl for NaCl at 50%, resulted in more selective growth of *L. plantarum* than in those with either KCl or NaCl alone. KCl was the only chloride substitute allowing growth of *E. cloacae*. Substitution of phosphates for NaCl at 50% enabled slow growth of *L. plantarum*.

INTRODUCTION

SALT (NaCl) is used in brines to direct the course of cucumber fermentation by greatly reducing the number of salt-sensitive, competitive, undesirable organisms and providing a selective environment for the desirable microorganisms in lactic acid fermentation (Fleming and McFeeters, 1981; Fleming, 1982).

The microbial distribution profile in raw cucumber indicates that lactic acid producing bacteria (LAB) occur in relatively low numbers ($5x10^3$CFU/g cucumber) as compared to the undesirable aerobic species ($1x10^7$CFU/g cucumbers (Etchells et al., 1973). Pederson (1979) reported the major aerobic species in raw vegetables were of the genera *Pseudomonas*, *Flavobacterium*, *Enterobacter*, *Escherichia*, and *Bacillus*.

Meneley and Stangehellini (1974) reported in certain healthy appearing cucumbers there were internally borne, soft rotting, plant pathogenic bacteria identified as members of the family *Enterobacteriaceae*. Further classification identified the isolates as *Proteus mirabilis*, *Citrobacter* species, *Enterobacter cloacae* and a yellow pigmented *Erwinia* species.

Samish et al. (1963) found that *Enterobacteriaceae* multiplied in both the fruit and brine during fermentation of green tomatoes. Ayres et al. (1980) reported *Enterobacter* species grew rapidly in cucumber fermentation at 5% initial brines, which reached maximum population within 2 days and vigorously produced carbon dioxide and hydrogen gases. Furthermore, they stated that at 15% salt concentrations, *Enterobacter* species could grow after a period of adaptation, achieving a maximum population in 12 to 14 days.

LAB previously found to be indigenous to cucumber fermentations were *Lactobacillus plantarum*, *Pediococcus cerevisiae*, *Leuconostoc mesenteroides*, *Streptococcus faecalis* and *Lactobacillus brevis* (Pederson and Albury, 1961). Pure culture inoculation studies of those microorganisms in cucumber fermentation indicated that regardless of inocula *Lactobacillus plantarum* completed all fermentation of cucumbers (Pederson and Albury, 1961).

Increasing concern about the relationship between sodium and development of hypertension in humans has prompted food processors to develop low or sodium-free products. The objectives of our study were: (1) to investigate partial or total replacement of NaCl in cucumber fermentation with phosphates, sulfates and/or other chloride salts and (2) to monitor the capability of non-toxic salts in cucumber fermentation to promote growth of a desirable microorganism, *Lactobacillus plantarum*, and suppress growth of an undesirable microorganism, *Enterobacter cloacae*.

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MATERIALS & METHODS

Cucumber extract preparation

Approximately 15 kg fresh cucumbers purchased from a commercial wholesaler were washed and extracted according to the procedure of Naewbanij et al. (1986). The supernatant was divided equally into six, 2-L portions and frozen. Frozen extract was thawed 2 hr at room temperature (21–25°C), then 24 hr at refrigerator temperature (5°C) before filtration. Extract was filtered through a Whatman 934-4H microfiber filter into sterile flasks.

Culture preparation

Lactobacillus plantarum (ATCC 8014), grown on MRS (Difco) agar medium, was transferred aseptically into MRS broth and *Enterobacter cloacae*, grown on nutrient (Difco) agar, into nutrient broth. Incubation conditions were 20–24 hr at 32°C for *L. plantarum* culture and 12–15 hrs at 32°C for *E. cloacae* before inoculation of the cucumber extract. Cultures were diluted so that a 1 mL inoculum would produce a concentration of approximately 10^7 CFU/mL.

Experimental procedures

Filtered cucumber extract: distilled water (80:18) was transferred into 250-mL sterile, screw-capped, Erlenmeyer flasks. Treatments included NaCl, ammonium chloride (NH_4Cl), ammonium phosphate ($NH_4H_2PO_4$), ammonium sulfate [$(NH_4)_2SO_4$], calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), magnesium sulfate ($MgSO_4$), potassium chloride (KCl), potassium phosphate (KH_2PO_4), potassium sulfate (K_2SO_4) and a salt-free control. Concentrations were 25%, 50% and 100% NaCl substitution based upon the ionic strength contribution to the total ionic strength = 1.0, of the brine (1.0 = the ionic strength of a 1M or 5.85% NaCl solution). The concentrations of different salts in various sets of experiments are shown in Table 1. A randomized complete block design with repeated measures was followed with duplicates for each level of substitution. Data collected for each experiment (25%, 50% or 100% NaCl substitution) were analyzed by analysis of variance. Least square means were determined when differences were significant.

Each flask containing extract was inoculated with 1mL each of *L. plantarum* and *E. cloacae* cultures grown as described, in at timed intervals. Samples were incubated immediately in a water bath at 30°C. Sampling to monitor fermentative changes was at 4 hr-intervals. Turbidity, pH, titratable acidity expressed as % lactic acid, reducing sugar, viable counts of lactobacilli and coliforms were monitored.

Turbidity, pH and titratable acidity measurements were made according to conditions and procedures previously cited by Naewbanij et al. (1986). Reducing sugars were analyzed by a modification of the Nelson (1944) method. Viable counts for lactobacilli were made by plating a serial dilution of samples on MRS, and coliforms on McConkey (Difco) agars. Plates were incubated at 32°C for 48 and 24 hrs, respectively. A growth curve was constructed for each organism under each set of conditions. Growth rate constants, generation time (min), and lag times for each microorganism were calculated from growth

Table 1—Concentrations of salts used for cucumber extract fermentation

Salt	Salt conc for 100% NaCl replacement		Salt conc for 50% NaCl replacement		Salt conc for 25% NaCl replacement	
	Molarity	Concentration (%)	Molarity	Concentration (%)	Molarity	Concentration (%)
Sodium chloride (NaCl)	1.00	5.85	0.50	2.92	0.75	1.46
Ammonium chloride (NH_4Cl)	1.00	5.34	0.50	2.67	0.25	1.34
Ammonium phosphate ($NH_4H_2PO_4$)	1.00	11.49	0.50	5.74	0.25	2.87
Ammonium sulfate ($(NH_4)_2SO_4$)	0.33	4.36	0.17	2.18	0.08	1.09
Calcium chloride ($CaCl_2$)	0.33	3.66	0.17	1.83	0.08	0.92
Magnesium chloride ($MgCl_2$)	0.33	3.14	0.17	1.57	0.08	0.78
Magnesium sulfate ($MgSO_4$)	0.25	3.08	0.12	1.54	0.06	0.77
Potassium chloride (KCl)	1.00	7.45	0.50	3.72	0.25	1.86
Potassium phosphate (KH_2PO_4)	1.00	13.60	0.50	6.80	0.25	3.40
Potassium sulfate (K_2SO_4)	0.33	5.75	0.17	2.88	0.08	1.44

Table 2—Means[a] for growth rate constants and lag times of Lactobacillus plantarum *in cucumber extract fermentation with various salt substitutes*

Salt	100% NaCl substitution		50% NaCl substitution		25% NaCl substitution	
	Growth rate constant (hr^{-1})	Lag time (hr)	Growth rate constant (hr^{-1})	Lag time (hr)	Growth rate constant (hr^{-1})	Lag time (hr)
NaCl	0.77c	12.15a	0.73c	11.90b	0.74ab	11.05b
NH_4Cl	0.57d	12.45a	0.54e	12.00b	0.52c	15.20b
$NH_4H_2PO_4$	N.G.[b]	N.G.	0.46f	18.60a	0.62b	21.40a
$(NH_4)_2SO_4$	1.27a	4.3 d	0.82bc	6.00c	0.67b	5.20cd
$CaCl_2$	0.79c	11.15b	0.62d	10.50b	0.60bc	10.00bc
$MgCl_2$	0.95b	10.35c	0.64d	9.15b	0.66b	5.00cd
$MgSO_4$	1.06b	4.2 d	0.63d	6.00c	0.83a	5.00cd
KCl	0.58d	12.5 a	0.77bc	1[illegible].40b	0.68b	13.10b
KH_2PO_4	N.G.	N.G.	0.49f	17.00a	0.62b	21.30a
K_2SO_4	1.08b	4.1 d	0.71c	4.90b	0.92a	5.10cd
Control (no salt)	0.87c	0 e	0.91a	0d	0.92a	0d

[a] Each value is a mean for two growth curves; means within column followed by same letters not significantly different ($P > 0.05$).
[b] N.G. = No growth

curves (Naewbanij et al. 1986). Data for each experiment were analyzed statistically by analysis of variance.

RESULTS & DISCUSSION

Growth parameters

Growth rate constants and lag times for *Lactobacillus plantarum* and *Enterobacter cloacae* grown in cucumber extracts with different salts and levels of NaCl substitution are presented in Tables 2 and 3. The shorter generation times required for *L. plantarum* to grow in sulfate salts than in NaCl or no salt media suggested that 100% NaCl substitution with sulfates had a stimulating effect on growth (Table 2). Similar effects were observed for *E. cloacae* (Table 3). Generation times for *L. plantarum* in KCl and in NH_4Cl generally were not different. However, generation times for *E. cloacae* in KCl brines were longer than in NaCl, indicating greater inhibition by KCl on the undesirable microorganism. No relationship appeared between initial "as-is" pH and generation time. Therefore, growth differences appeared attributable to effects of salt type.

Sulfates stimulated growth of both microorganisms at 100% substitution, whereas phosphates completely suppressed microbial growth. Some chloride salts (NH_4Cl, $MgCl_2$ and $CaCl_2$) were completely inhibitory to *E. cloacae*, although KCl and NaCl were not. When generation times for *L. plantarum* increased, times for *E. cloacae* also tended to increase if microbial growth occurred under those conditions (Tables 2 and 3).

Replacing 50% of the NaCl with other salts resulted in less discrepancy between sulfate and chloride salts in generation times for *L. plantarum*. However, lag times for both organisms grown in chloride brines were longer than when grown in sulfate brines (Tables 2 and 3). Results with 50% NaCl substitution by KCl, however, suggested those conditions became selective for LAB, as indicated by decreased generation time for *L. plantarum* and increased generation time for *E. cloacae*. With sulfates, generation times for *L. plantarum* were higher than for *E. cloacae*, suggesting those conditions were more favorable for growth of *E. cloacae*.

A generation time of 46 min obtained for *L. plantarum* grown without salt at the usual pH of cucumber extract (pH 5.7) approximated the 43 min generation time for that organism grown in cabbage juice at pH 6.2 (Stamer et al., 1971). Partial replacement of NaCl at 50% or 25% with other salts in cucumber brines generally increased generation times for both organisms suggesting NaCl had on an inhibitory influence on growth. Environments containing NH_4Cl, $MgCl_2$, $CaCl_2$, KH_2PO_4 and $NH_4H_2PO_4$ at all concentrations we used were inhibitory to growth of *E. cloacae*. Generation times for *L. plantarum* increased as NaCl substitution decreased, except for K_2SO_4, KH_2PO_4 and $NH_4H_2PO_4$. Generation times were higher for those salts when higher NaCl substitutions were made (Table 2).

The slight difference in generation times for *L. plantarum* and *E. cloacae* in the NaCl environment suggested NaCl did not provide selective conditions for LAB although it was partly inhibitory to both microorganisms. Other chloride salts such as NH_4Cl, $CaCl_2$ and $MgCl_2$ were more selective, since they completely inhibited growth of *E. cloacae*. Substitution of KCl for NaCl at 50% provided more selective conditions for *L. plantarum* than when NaCl was replaced at 100% or 25% in the brines. Likewise, 50% NaCl substitution by NH_4Cl hastened growth of *L. plantarum* more than when substitutions of 25% or 100% were made. Conversely, $MgCl_2$ or $CaCl_2$ was more favorable for growth of *L. plantarum* when substituted for NaCl completely rather than partly. Thus, under microaerophilic conditions, most chloride salts provided selective conditions for growth of LAB.

Fermentative changes

Titratable acidity, pH and reducing sugar changes in brines with various salts 100% substituted for NaCl are shown in Table 4. Although collected at 4-hr intervals data are reported only at 12 hr intervals. Growth of microorganisms was retarded greatly by phosphate salts which enhanced buffering. Since phosphates would not be acceptable substitutes for NaCl in

Table 3—Means[a] for growth rate constants and lag times of Enterobacter cloacae *in cucumber extract fermentation with various salt substitutes*

Salt	100% NaCl substitution		50% NaCl substitution		25% NaCl substitution	
	Growth rate constant (hr^{-1})	Lag time (hr)	Growth rate constant (hr^{-1})	Lag time (hr)	Growth rate constant (hr^{-1})	Lag time (hr)
NaCl	0.78ab	12.00a	0.84c	20.00a	0.80a	15.00a
NH_4Cl	N.G.[b]	N.G.	N.G.	N.G.	N.G.	N.G.
$NH_4H_2PO_4$	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
$(NH_4)_2SO_4$	1.00a	4.00b	0.93b	3.90c	0.82a	4.00b
$CaCl_2$	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
$MgCl_2$	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
$MgSO_4$	1.12a	4.00b	1.18a	4.00c	0.80a	4.00b
KCl	0.60b	10.00a	0.49d	10.00b	0.78a	14.00a
KH_2PO_4	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
K_2SO_4	1.14a	0.40b	0.85c	3.90c	0.78a	3.80b
Control (no salt)	1.00a	0c	1.00b	0d	0.93a	0b

[a] Each value is a mean for two growth curves; means within column followed by same letters not significantly different ($P>0.05$).
[b] N.G. = No growth

Table 4—pH, titratable acidity, and total reducing sugars in cucumber extract fermentation brines inoculated with L. plantarum *and* E. cloacae *with 100% NaCl substitution.*[a]

Fermentation time (hr)	Salt substitute								No salt (Control)
	NaCl	NH_4Cl	$(NH_4)_2SO_4$	$CaCl_2$	$MgCl_2$	$MgSO_4$	KCl	K_2SO_4	
				pH Values					
0	5.38	5.41	5.70	4.79	4.68	4.96	5.61	5.76	5.63
12	5.29	5.39	5.44	4.58	4.56	4.96	5.50	5.48	5.26
24	4.94	4.90	4.04	3.35	3.25	3.62	4.13	4.09	3.86
36	4.20	3.71	3.74	3.19	3.06	3.32	3.74	3.76	3.59
48	3.94	3.61	3.70	3.10	3.05	3.29	3.72	3.74	3.54
				Titratable acidity[b]					
0	0.08	0.70	0.40	0.14	0.09	0.12	0.07	0.08	0.18
12	0.10	1.06	0.61	0.15	0.17	0.23	0.09	0.15	0.22
24	0.12	1.07	1.02	0.27	0.28	0.48	0.21	0.44	0.51
36	0.26	1.37	1.22	0.36	0.48	0.74	0.38	0.68	0.82
48	0.30	1.12	1.19	0.37	0.48	0.79	0.36	0.76	0.85
				Reducing sugars[c]					
0	2.31	2.25	2.30	2.36	2.29	2.30	2.29	2.29	2.29
12	2.29	2.17	1.12	2.18	2.14	1.26	2.06	0.98	0.99
24	2.00	1.68	0.70	1.91	1.54	0.83	1.56	0.59	0.70
36	1.33	1.60	0.40	1.76	1.58	0.50	1.59	0.23	0.26
48	1.28	1.48	0.21	1.64	1.47	0.50	1.61	0.13	0.15

[a] Means for four determinations.
[b] Percentage lactic acid.
[c] Percentage based on volume of brine analyzed.

Table 5—pH values, titratable acidity, and total reducing sugars for cucumber extract fermentation brines inoculated with L. plantarum *and* E. cloacae *with 50% NaCl substitution.*[a]

Fermentation time (hr)	Salt substitute								No salt (Control)
	NaCl	NH_4Cl	$(NH_4)_2SO_4$	$CaCl_2$	$MgCl_2$	$MgSO_4$	KCl	K_2SO_4	
				pH Values					
0	5.17	5.42	5.70	5.00	5.03	5.16	5.53	5.60	5.65
12	5.31	5.30	4.94	4.72	4.81	4.59	5.37	4.86	4.77
24	5.11	4.41	3.94	3.59	3.64	3.62	4.12	3.90	3.82
36	3.97	3.86	3.67	3.47	3.46	3.36	3.84	3.64	3.60
48	3.56	3.60	3.52	3.28	3.30	3.24	3.61	3.51	3.50
				Titratable acidity[b]					
0	0.07	0.10	0.20	0.08	0.43	0.08	0.08	0.05	0.10
12	0.08	0.14	0.53	0.13	0.60	0.16	0.08	0.18	0.30
24	0.14	0.25	0.55	0.21	0.72	0.40	0.20	0.40	0.59
36	0.26	0.30	0.83	0.34	0.60	0.49	0.30	0.57	0.80
48	0.57	0.36	0.84	0.39	0.68	0.54	0.39	0.65	0.88
				Reducing sugars[c]					
0	2.25	2.21	2.35	2.21	2.14	2.32	2.21	2.29	2.30
12	2.17	2.19	1.76	1.99	2.17	1.76	2.18	1.78	1.17
24	2.01	1.81	1.28	1.81	1.84	1.21	1.78	1.21	0.75
36	1.58	1.76	1.04	1.65	1.71	0.90	1.63	1.01	0.34
48	1.32	1.62	0.80	1.49	1.56	0.71	1.41	0.84	0.10

[a] Means for four determinations.
[b] Percentage lactic acid.
[c] Percentage based on volume of brine analyzed.

cucumber fermentation, these data are not presented. Notable observations were variable initial inherent acidities, unchanging pH and titratable acidities of the brines with phosphate salts, and higher growth rates in those with sulfate salts (Table 3) when compared to NaCl. These changes corresponded to microbial growth patterns for various brines. Faster growth of *L. plantarum* and *E. cloacae* in sulfate brines (Tables 2 and 3) accounted for faster rates of fermentation which reduced sugars in cucumber extracts (Table 4) into lactic acid and other undetermined fermentation products. Faster and greater production of lactic acid in brines with sulfates also indicated fermentative activities. Final pH, however, appeared related

Table 6—pH values, titratable acidity, and total reducing sugars for cucumber extract fermentation brines inoculated with L. plantarum *and* E. cloacae *with 25% NaCl substitution.*[a]

Fermentation time (hr)	Salt substitute								No salt (Control)
	NaCl	NH_4Cl	$(NH_4)_2SO_4$	$CaCl_2$	$MgCl_2$	$MgSO_4$	KCl	K_2SO_4	
				pH Values					
0	5.09	5.12	5.18	4.85	4.73	4.96	5.15	5.20	5.53
12	5.13	5.17	5.20	4.92	4.80	4.80	5.21	5.24	5.41
24	5.13	4.88	4.28	4.06	4.32	4.00	4.51	4.22	3.97
36	3.99	3.84	3.76	3.55	4.08	3.59	3.80	3.76	3.74
48	3.69	3.64	3.61	3.43	3.53	3.44	3.66	3.61	3.64
				Titratable acidity[b]					
0	0.08	0.28	0.24	0.10	0.09	0.08	0.07	0.08	0.08
12	0.12	0.35	0.38	0.14	0.12	0.12	0.09	0.09	0.25
24	0.15	0.54	0.45	0.22	0.16	0.24	0.18	0.26	0.58
36	0.23	0.65	0.53	0.31	0.27	0.32	0.25	0.34	0.69
48	0.30	0.49	0.51	0.42	0.31	0.28	0.31	0.40	0.81
				Reducing sugars[c]					
0	2.10	2.08	2.21	2.08	2.10	2.03	2.03	2.21	2.11
12	1.92	1.87	1.88	1.93	1.83	1.96	1.92	1.94	1.15
24	1.81	1.60	1.54	1.65	1.76	1.48	1.59	1.40	0.62
36	1.51	1.48	1.26	1.56	1.59	1.37	1.47	1.28	0.33
48	1.41	1.28	1.14	1.45	1.53	1.19	1.41	1.15	0.13

[a] Means for four determinations.
[b] Percentage lactic acid.
[c] Percentage based on volume of brine analyzed.

to initial pH of brines (Table 4). Those with lower initial pH tended to attain lower final pH, with exception of brines with phosphates which showed no change in pH.

Titratable acidity, pH, and reducing sugar changes in brines containing various salts at 50% NaCl substitution are shown in Table 5. Notable differences in fermentative changes at 50% substitution compared with those replacing NaCl completely were: final pH of brines at 50% substitution generally were higher except in brines with phosphates in which final pH was lower. In contrast, NaCl tended to suppress bacterial growth in sulfate and other chloride brines (Tables 2 and 3). Final concentrations of reducing sugars in those extracts (Table 5) also indicated lower fermentative activities.

In brines with 25% NaCl substitution, final pH after 48-hr fermentation (Table 6) was higher than that in brines with 50% or 100% NaCl substitution. Concentrations of titratable acidity and reducing sugars (Table 6) in 48-hr fermentation brines were almost the same, except for brines with sulfates, in which fewer reducing sugars were fermented. Less lactic acid was produced in brines with 25% NaCl substitution than in those with 50% substitution.

These results suggested that among groups of salts tested, chlorides provided the most selective conditions for growth of the desirable microorganism, *L. plantarum*, in cucumber fermentation. Concentrations of NaCl substitution by other chloride salts did not appear critical in the microbial selectivity except for KCl and NH_4Cl. Substitutions by those salts for NaCl at 50% promoted growth of *L. plantarum* better than their substitution at 25%.

CONCLUSIONS

SULFATES generally stimulated growth of both *L. plantarum* and *E. cloacae* when substituted at 100% in fermenting cucumber brines. Phosphates completely suppressed microbial growth. NH_4Cl, $MgCl_2$, and $CaCl_2$ were completely inhibitory to *E. cloacae*, but KCl and NaCl were not. Generation times increased for microorganisms when salts were used at 50% NaCl substitution. Substitution of sulfate for NaCl generally increased generation times for both microorganisms but enabled growth of *E. cloacae* more than *L. plantarum*. Substitution of NaCl by 50% KCl, resulted in conditions more selective for growth of *L. plantarum* than brines containing either KCl or NaCl alone. Substitution of phosphates for NaCl at 50% enabled relatively slow growth of *L. plantarum*. No general trends were observed at 25% NaCl substitution, except that fermentative activities were lessened. Higher final pH and more nonfermented reducing sugars resulted with 25% substitution. All chloride salts tested provided more selective conditions for *L. plantarum* than NaCl.

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Role of Noncovalent Forces in Micellization Using Legumin from *Vicia faba* as a Study System

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ABSTRACT

The seed storage protein, legumin, from *Vicia faba* interacted to form micelles and elaborate protein networks under varying conditions. Several molecular parameters correlated with observed micelle interaction parameters; these included thermal properties as indicators of protein stability and surface hydrophobicity as an assessment for potential hydrophobic interactions. The optimal pH for micelle formation ranged from 5.5 to 6.5, values at which electrostatic repulsions were minimal and surface hydrophobicites were adequate to allow hydrophobic interactions. The micelle response was affected by anions both in terms of concentration and type of anion. Finally, gradual denaturation of legumin with increasing urea concentrations had a negative impact on the micelle response.

INTRODUCTION

UNDER SPECIFIC environmental conditions, the fababean (*Vicia faba*) storage protein vicilin has the capacity to self-associate into a stable micelle arrangement. This micelle formation is a valuable method for protein isolation (Murray et al., 1978) and it also is a preliminary stage in elaborate protein network establishment (Ismond et al., 1986a, b). On that basis, it was important to determine whether this is a unique vicilin response or whether it is common to other seed storage globular proteins. Our study was designed to extend this micellization using legumin, the 11S globular storage protein form *Vicia faba*, as a study system. The micelle forming capacity for legumin was examined under different pH, electrolyte and denaturing conditions and correlated with several molecular parameters including thermal stability and surface hydrophobicity. The ultimate goal was to demonstrate that micellization can be predicted and manipulated by controlling electrostatic and hydrophobic noncovalent interactions.

MATERIALS & METHODS

Protein isolation

Legumin was isolated form *Vicia faba* according to the method of Georgiou (1987).

Environmental influence on legumin interaction

To establish the influence of various environmental conditions on protein micelle formation and interactions, legumin (approximately 1 mg mL^{-1}) was exposed by extensive dialysis to a series of environmental conditions given in Table 1. While these environments were chosen to evaluate electrostatic and hydrophobic forces as well as conformational change, the conditions were limited tc those in which legumin was soluble. The most obvious problem with this approach was that it was impossible to establish values for controls (i.e. legumin in water only) for either the salt series or the urea series. For each environment examined, the capacity of legumin to form micelles was assessed using the standard procedure of Ismond et al. (1985). Protein samples were initially concentrated to 75 mg mL^{-1} by ultrafiltration. The samples were subsequently examined without a coverslip using a Zeiss Universal Research Microscope. Distilled water (20 μL) was then added to the protein solution (20 μL). After precipitation, a coverslip was added. The micelle response was rated from 0 to 5 according to a scheme developed by Ismond et al. (1986a; Fig. 1).

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Protein characterization

The thermal properties of legumin in each environment were determined using a DuPont 9900 thermal analysis system with a 910 Differential cell base, and a pressure DSC cell, as described by Arntfield and Murray (1981). Sample preparation and running conditions were given in Ismond et al. (1985).

In addition, the aliphatic surface hydrophobicity (S_c) of legumin exposed to each electrolyte was determined using the method of Kato and Nakai (1980) with cis-parinaric acid (Calbiochem-Behring Corp.) as a fluorescence probe. Aromatic surface hydrophobicity (Sa) of legumin was determined according to the method of Hayakawa and Nakai (1985) using 1-anilino-8-napthalene (ANS; Sigma Chem. Co.) as an extrinsic fluorescence probe. In both cases, the relative fluorescence intensity (RFI) was measured with a Perkin-Elmer LS-5 fluorescence spectrophotometer using a slit width of 0.5nm and a fixed scale of 1.0.

Protein concentrations for DSC and fluorescence analyses were determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA; Sigma Chem. Co.) as a standard. Levels of legumin in $NaC_2H_3O_2$ and NaSCN were assessed using Coomassie Blue R-250 reagent (Pierce Chem. Co.) to avoid possible anionic interference with the Lowry reaction. All thermal parameters plus S_c and S_a values were determined using a minimum of four samples; means and standard deviations of the mean were calculated for each.

RESULTS & DISCUSSION

SPECIFIC PROTEINS, in spite of conformational limitations, have the capacity to self-associate into a micelle arrangement under certain environmental conditions (Murray et al., 1978; Simons et al., 1978; Evans and Phillips, 1979; Ismond et al., 1986a, b). A major factor in this micelle response is the environment. Amphiphilic protein molecules are dynamic and flexible; conformational changes can occur with minor environmental fluctuations (Cooper, 1980). In order to achieve a micelle arrangement, a specific intermolecular hydrophilic-hydrophobic balance apparently is required (Tanford, 1973). Slight environmental modifications may alter this balance such that micelle formation no longer occurs.

Earlier studies have indicated that self-association into a micelle arrangement may be attributed to protein hydrophobic interactions (Murray et al., 1978; Ismond et al., 1986a, b). Recognizing that, we initially assumed the self-associating

Table 1—Legumin environments used for micelle assessment, differential scanning calorimetery and fluorescence spectroscopy

Solution	Environment
Group A	pH values
phosphate buffer (0.25M)	5.5, 6.0, 6.5, 7.0
	7.5, 8.0, 8.5, 9.0
Group B	
(in 0.01M phosphate buffer, pH 6.5)	Electrolyte conc (μ)
NaCl[a,b]	0.25, 0.5, 1.0
$NaC_2H_3O_2$[b]	0.25, 0.5, 1.0
NaSCN[b]	0.25, 0.5, 1.0
$Na_3C_6H_5O_7$[b]	0.25, 0.5, 1.0
Group C	
(in 0.01M phosphate buffer, pH 6.5)	Urea conc (M)
urea	0.5, 1.0, 2.0
	3.0, 6.0, 8.0

[a] Sodium chloride not used in fluorescence experiments due to quenching by the anion.

[b] NaCl-sodium chloride, $NaC_2H_3O_2$-sodium acetate, NaSCN-sodium thiocyanate, $Na_3C_6H_5O_4$-sodium citrate.

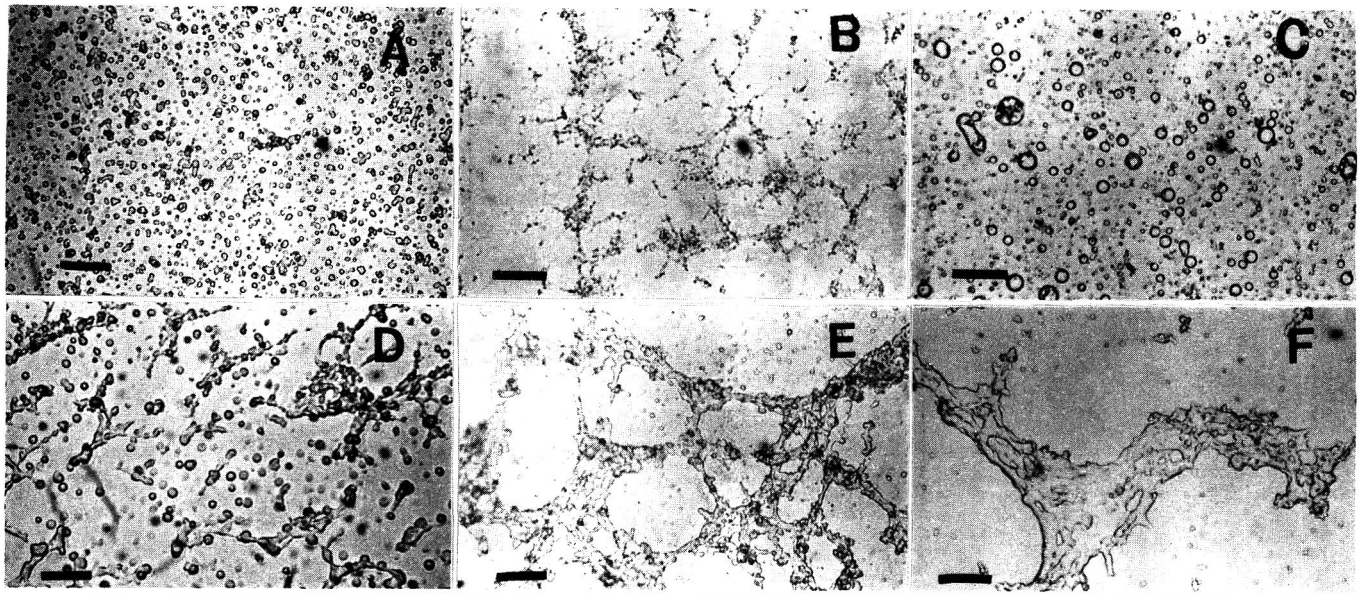

Fig. 1—Photomicrographs of micelle responses corresponding to ratings 1 through 5. Bar represents 25 μm. (A) Small single micelles, rating 1; (B) Small micelles in aggregates, rating 2; (C) Small, intermediate and large micelles, rating 3; (D) Homogenous coalescence, rating 4; (E,F.) Extensive coalescence, rating 5.

molecules would be highly hydrophobic in nature. However, in comparison with other globular proteins described by Bigelow (1967), fababean legumin, a molecule capable of extensive micelle formation, exhibited a low Bigelow hydrophobicity (819.46 cal/a.a. residue) and a high charge frequency (0.46). This was similar to the situation with vicilin, another fababean storage protein (Ismond, 1984). In addition, legumin was characterized by a relatively moderate surface hydrophobicity (S_o) – both in terms of aliphatic (S_c = 44.7 to 142.0) and aromatic (S_a = 42.6 to 561.6) values. Although the low Bigelow hydrophobicity values for legumin seem related to the moderate S_o values, there was no correlation between these two parameters for globular proteins in general (Nakai, 1983).

As a result of these low hydrophobicity values legumin did not appear initially as an ideal protein for micelle formation. However, we must point out that a definite intramolecular hydrophobic-hydrophilic balance is necessary for micelle formation (Tanford, 1973). For example, Simons et al. (1978) suggested that bacteriorhodopsin, an integral membrane protein, would not aggregate into a micelle structure due to its high molecular hydrophobicity. With extreme hydrophobicity, protein molecules could not form sperical aggregates with hydrophilic surfaces extensive enough to retain a micelle arrangement. Thus, an appropriate molecule for micelle formation would appear to be one similar to legumin in which there was a specific intramolecular balance of hydrophilic and hydrophobic residues. However, the surface balance depends on the nature of the environment; the flexible conformation of a dynamic protein means the critical balance could be disrupted with minimal changes in the environment.

Types of micelle responses with legumin

From examination of micelle formation by legumin in a number of different environments, legumin could apparently self-aggregate into a variety of forms from single micelles to extensive molecular networks. Self-aggregation into a population of discrete micelles is considered to be a thermodynamically favorable event, resulting from intermolecular hydrophobic interactions (ratings 1, 2 and 3; Fig. 1). The stabilization of a static population of discrete micelles is probably the result of cooperative interaction of noncovalent forces. According to Tanford (1973), attractive forces must be dominant in order to restrict the micelles to a specific size. For legumin, the environmental media had a definite impact on development of this surface repulsion.

In some situations, avoiding development of a major repulsive situation was possible (rating 5; Fig. 1). Interaction continued until extensive amorphous masses of protein occurred with complete phase separation. This type of reaction required a microenvironment established around individual micelles with minimized repulsive forces and maximized attractive interactions.

In other cases, there was an intermediate association (rating 4; Fig. 1). Discrete micelles were followed by some coalescence which reached a static end-point without continuing on to development of amorphous protein masses. At some point in the interaction, repulsive electrostatic forces dominated such that possible hydrophobic associations were minimized.

Effect of pH on micelle formation by legumin

From the micelle observations, the optimum structural characteristics of legumin for micelle formation and interaction existed from pH 5.5 to 6.5 with some diminishing in response at pH 6.5 (Table 2). From pH 7.0 to 9.0, however, there was a complete absence of micelle response. At pH 7.0, both aliphatic and aromatic (S_c and S_a) hydrophobicity values decreased significantly from those at lower pH levels. From the

Table 2—Micelle rating (MR), aliphatic surface hydrophobicity (S_c), aromatic surface hydrophobicity (Sa), denaturation temperature (Td) and enthalpy of denaturation (ΔH) for legumin in 0.25M phosphate buffer of different pH values

pH	MR[d]	S_c	S_a	Td (°C)	ΔH (cal/g)
5.5	5	115.3 ± 9.0[a]	157.0 ± 10.6	106.9 ± 0.6[ab]	3.30 ± 0.21[a]
6.0	5	130.8 ± 8.6[b]	193.0 ± 11.8	106.2 ± 0.7[ab]	2.78 ± 0.11
6.5	4	109.3 ± 8.0[ac]	119.6 ± 7.9[a]	106.3 ± 0.7[ab]	3.34 ± 0.48[a]
7.0	0	76.7 ± 9.5	105.9 ± 8.9[a]	107.7 ± 0.3[ab]	3.27 ± 0.67[a]
7.5	0	114.6 ± 5.2[a]	561.7 ± 30.4[b]	108.3 ± 1.0[b]	3.46 ± 0.38[a]
8.0	0	134.3 ± 11.6[b]	537.7 ± 29.8[b]	106.1 ± 0.6[ab]	3.32 ± 0.40[a]
8.5	0	100.5 ± 5.3[ac]	471.0 ± 89.9[bc]	105.2 ± 1.1[a]	3.15 ± 0.30[a]
9.0	0	100.5 ± 9.8[ac]	356.4 ± 37.6[c]	105.1 ± 3.2[a]	3.06 ± 0.19[a]

[a-c] Column values followed by the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

[d] Description of micelle ratings is given in Fig. 1.

Table 3—Temperature of denaturation (Td) and enthalpy of denaturation (ΔH) values for legumin in various concentrations of different sodium salts

Salt environment	Concentration (M) 0.25	0.5	1.0
	Td (°C)		
Control(0.0M)	103.1 ± 0.1[a1]	103.1 ± 0.1[a1]	103.1 ± 0.1[1]
NaSCN	98.6 ± 2.9[a1]	97.9 ± 1.3[1]	95.4 ± 1.4[1]
$NaC_2H_3O_2$	106.3 ± 2.5[b1]	109.0 ± 0.7[b1]	112.7 ± 0.1[a]
NaCl	105.8 ± 0.1[b]	108.6 ± 0.4[b]	114.1 ± 0.3[a]
$Na_3C_6H_5O_7$	103.3 ± 0.4[ab1]	103.3 ± 0.1[a1]	105.7 ± 0.2
	ΔH (cal/g)		
Control(0.0M)	3.44 ± 0.10[1]	3.44 ± 0.10[a1]	3.44 ± 0.10[a1]
NaSCN	3.62 ± 0.05[1]	3.67 ± 0.15[a1]	2.43 ± 0.12
$NaC_2H_3O_2$	4.22 ± 0.09[a1]	4.22 ± 1.22[ab12]	5.45 ± 0.18[2]
NaCl	4.56 ± 0.46[a]	5.19 ± 0.13[b]	6.43 ± 0.17
$Na_3C_6H_5O_7$	3.18 ± 0.21[1]	2.89 ± 0.40[a]	3.31 ± 0.10[a1]

a,b Column values followed by same letter not significantly different by the Student-Newman-Keuls test ($P \leq 0.05$). Statistical comparisons apply to single paramenter at one concentration level.

1,2 Row values followed by same number not significantly different by the Student-Newman-Keuls test ($P \leq 0.05$). Statistical comparisons apply to single parameter in one specific salt.

Table 4—Micelle rating (MR), aliphatic surface hydrophobicity (Sc) and aromatic surface hydrophobicity (Sa) values for legumin in various concentrations of different sodium salts[x]

Salt environment	Concentration (M) 0.25	0.5	1.0
	MR[y]		
NaSCN	5	5	2
$NaC_2H_3O_2$	5	5	2
NaCl	5	5	2
$Na_3C_6H_5O_7$	2	2	0
	S_c		
NaSCN	50.9 ± 7.6	99.0 ± 20.4[a]	124.7 ± 33.7[a]
$NaC_2H_3O_2$	142.0 ± 35.0[a1]	89.7 ± 33.4[ab12]	74.8 ± 29.4[ab2]
$Na_3C_6H_5O_7$	107.7 ± 15.7[a]	59.5 ± 11.8[b1]	44.7 ± 13.7[b1]
	S_a		
NaSCN	42.7 ± 4.1	82.4 ± 10.9[a]	123.6 ± 14.9
$NaC_2H_3O_2$	115.1 ± 20.6[1]	111.5 ± 26.5[a1]	76.3 ± 23.9[1]
$Na_3C_6H_5O_7$	466.0 ± 71.9[1]	423.1 ± 30.0[1]	264.2 ± 41.3

[x] No control salt concentration (0.0M) included due to problem of legumin solubility. No S_c and S_a values determined with NaCl due to quenching effect of the anion.

[y] Description of micelle rating shown in Fig. 1.

a,b Column values followed by same letter not significantly different by the Student-Newman-Keuls test ($P \leq 0.05$). Statistical comparisons apply to single paramenter one concentration.

1,2 Row values followed by same number not significantly different by the Student-Newman-Keuls test ($P \leq 0.05$). Statistical comparisons apply to single parameter in one specific salt.

results legumin conformation appeared not ideal for micelle formation if hydrophobic interactions were suppressed due to decrease in S_c and S_a values. The observed importance of surface hydrophobicity in general was supportive of the original premise by Murray *et al.* (1978) that these micelle structures were products of hydrophobic associative forces.

In contrast to the surface hydrophobicity values at pH 7.0, both S_c and S_a values significantly increased at pH 7.5 (Table 2). In fact, the S_a reached the highest value observed in the pH study. The absence of the micelle response at this pH may be explained in terms of a critical hydrophilic-hydrophobic balance. As the pH was increased, the protein became more electronegative; as a result of increased electrostatic repulsion, the protein may have undergone conformational change. Interestingly, this change was not sufficient to be reflected in protein thermal parameters (Td, ΔH; Table 2). In fact, the stable Td and ΔH values over the pH range precluded any major stuctural alterations.

As the pH was raised from 7.5 to 9.0, the micelle response was still not observed. This was similar to results reported for vicilin from *Vicia faba* (Ismond et al., 1986a). Several observations could be made with respect to these pH values. Firstly, the S_a values were still high, possibly resulting in an unfavorable shift in hydrophilic - hydrophobic balance. Secondly, the overall negativity became increasingly important in the micelle response. In addition to increasing overal protein negative charge, the electrolyte environment changed with an increase in pH. As the pH was changed form 7.5 to 9.0, the balance between univalent phosphate ($H_2PO_4^-$) and divalent phosphate (HPO_4^{-2}) would be expected to shift from a predominant $H_2PO_4^-$ at pH 6.0 to a predominant HPO_4^{-2} at pH 8.0. As a result of the presence of the divalent anion, the system of protein and solvent became more electronegative as pH increased to pH 9.0. This extensive electrostatic repulsion was not conducive to hydrophobic associations and subsequent micelle formation.

Effects of Specific Salts on Legumin Stability and Micelle Forming Capacity

In general, effects of different electrolytes on protein-protein interactions have been attributed to variations in the anion (Robinson and Jencks, 1965). This was a result of the different hydration phenomena. With cations, the extensive hydration shells result in their almost complete exclusion from the water layer at the protein interface. In contrast, anions are quite variable in extent of exclusion due to their limited hydration spheres (Eagland, 1975). With respect to anions, the importance of both the identity and the concentration of the anion is acknowledged in assessing protein conformational parameters. At low concentrations ($\mu < 0.5$), anions may be considered as a collective group with effects attributed to electrostatic interactions related to the polar polyionic nature of the protein (von Hippel and Schliech, 1969). Most anions, therefore, at low concentrations have a "salting-in" effect with the extent of the electrostatic influence related to the ionic strength of the salt as well as the distribution and density of charged groups on the protein surface (von Hippel and Schliech, 1969).

In our study, however, at $\mu = 0.25$, impact of the different anions on both conformational properties and micelle-forming capabilities varied. The Td values for control legumin or legumin in 0.25 M NaSCN were significantly lower than those for legumin in NaCl or $NaC_2H_3O_2$ (Table 3). The destabilizing influence of SCN^-, apparent at low concentrations of the anion, may be related to its capacity for a high degree of nonspecific binding to a number of exposed protein polar sites (Arakawa and Timasheff, 1982). Similar results were obtained for vicilin from *Vicia faba* (Ismond et al., 1986b).

For the three anions, SCN^-, Cl^- and $C_2H_3O_2^-$, the micelle reaction was extensive (rating 5; Fig. 1). In these situations, the sudden dilution of concentrated legumin solution may have resulted in the physical disturbance of the electrical double layer associated with the protein molecules. Therefore, intermolecular association of legumin by hydrophobic associations would be thermodynamically favorable in response to the aqueous environment if the protein surface hydrophobicity were adequate. As a further consequence of the dilution, electrostatic effects on the micelle surface may have been minimized to reduce intermicelle repulsive forces such that further interactions were favored.

For $Na_3C_6H_5O_7$ ($\mu = 0.25$; Table 4), the micelle response was reduced (rating 2) in comparison with the other three media. This may be related to the high aromatic hydrophobicity (S_a) observed - a value of 466.0 for $C_6H_5O_7^{-3}$ as compared to 115.0 and 42.6 for $C_2H_3O_2^-$ and SCN^-, respectively. The exaggerated hydrophobicity may not be favorable for extensive micelle formation, as discussed previously. The extensive exposure of originally internal hydrophobic side-chains may disturb the orientation of the hydrophobic-hydrophilic residues such that micelle arrangement is more difficult.

As the salt concentration was increased to higher levels ($\mu > 0.5$), the identity of the anion became more important whereas the protein charge status became less significant. With

Table 5—Micelle rating (MR), aliphatic surface hydrophobicity (S_c), aromatic surface hydrophobicity (S_a), denaturation temperature (Td) and enthalpy of denaturation (△H) for legumin in different urea concentrations (M)

Conc M	MR[c]	S_c	S_a	Td (°C)	△H (cal/g)
0.0	–	–[d]	–[d]	103.1 ± 0.1	3.44 ± 0.10[a]
0.5	5	139.5 ± 1.1[a]	366.5 ± 27.6[a]	108.6 ± 0.1	3.40 ± 0.33[a]
1.0	2	130.7 ± 23.7[ab]	336.5 ± 27.1[a]	106.1 ± 0.3[a]	3.35 ± 0.18[a]
2.0	2	108.1 ± 21.6[b]	229.9 ± 45.1[a]	106.2 ± 0.1[a]	3.87 ± 0.07
3.0	2	67.5 ± 5.6	175.9 ± 14.0[b]	102.4 ± 0.2	3.06 ± 0.16[a]
6.0	1	49.0 ± 9.9	33.7 ± 9.8	94.3 ± 0.6	0.38 ± 0.08
8.0	1	31.7 ± 8.4	12.0 ± 4.9	87.1 ± 0.8	0.02 ± 0.00

[a,b] Column values followed by same letter not significantly different by the Student-Newman-Keuls test ($P \leq 0.05$).
[c] Description of micelle ratings shown in Fig. 1.
[d] Values not available due to insolubility of legumin.

respect to charge at high salt levels, electrostatic interactions between charged residues were effectively neutralized by extensive ionic shielding (Kirkwood, 1943). As a result, the protein behaved as a neutral dipole; general anionic electrostatic influences became less important. At high concentration levels, anions in general can be divided into two categories — stabilizing and destabilizing. The latter tend to promote protein solubility, and increasing destabilization, as salt concentrations are increased. On the other hand, stabilizing anions tend to stabilize conformational properties; at high concentrations, these anions cause protein precipitation as a consequence of hydrophobic associations (von Hippel and Wong, 1964). The mechanism of action of the two types of salts has received considerable attention. In general, the effect of a stabilizing anion on protein structure seems related to causing preferential hydration of the protein surface (Arakawa and Timasheff, 1982) in addition to its capacity to increase surface tension of the protein environment (Melander and Horvath, 1977). Therefore, as the concentration of the salt was increased, the salt-water interaction resulted in a gradual elimination of the salt from the protein surface. This effect resulted in an environment in which it is thermodynamically unfavorable for the protein to unfold and expose hydrophobic residues. In general this is referred to as a lyotropic effect. In our study $C_6H_5O_7^{-3}$, $C_2H_3O_2^-$ and Cl^- fell into the stabilizing category.

In contrast, destabilizing anions are characterized by a reduced capacity to increase surface tension of water (Melander and Horvath, 1977) and consequently, by less inducing of protein preferential hydration (Arakawa and Timasheff, 1982). In fact, destabilizing anions appear to remain bound to the protein surface even at high concentrations (Bull and Breese, 1970; Arakawa and Timasheff, 1982). This preferential binding results in increased electrostatic disturbances followed by conformational destabilization. In our study SCN^- was considered a destabilizing anion.

For the stabilizing salts, there was a positive relationship between the thermal stability of legumin, as assessed by Td values, and the concentration of various anions in comparison with values for NaSCN (Table 3). Using Td and H values as guidelines, the relative effectiveness of the anions could be described as:

$$Cl^-, C_2H_3O_2^- > C_3H_5O_7^{-3}$$

This differs from the original Hoffmeister series (1888) and that suggested by more recent researchers (Robinson and Jencks, 1965). In those studies, $C_6H_5O_7^{-3}$ was recognized as a highly stabilizing anion with Cl^- and $C_2H_3O_2^-$ as moderately stabilizing anions.

With SCN^-, both Td and △H values significantly decreased as salt concentration increased to $\mu = 1.0$ (Table 3). In addition, surface hydrophobicity significantly increased (both S_a and S_c) with an increase in SCN^- concentration (Table 4). This may reflect the increased exposure of hydrophobic residues as a result of extensive electrostatic disturbances with SCN^- binding to the surface of legumin.

The high salt levels influenced the capacity of legumin to self-associate into micelles. At $\mu = 0.5$, micelle interactions were identical to those at $\mu = 0.25$ (Table 4); in contrast, the individual responses significantly deteriorated at $\mu = 1.0$ (Table 4). Although decreases in micelle response were observed for each salt, the actual mechanisms causing the decrease may vary with individual environments. With SCN^-, the anion remained bound to the protein surface (Arakawa and Timasheff, 1982); this binding may persist after dilution to cause electrostatic repulsion or it may resume after dilution to create a charge repulsion among established micelles. In relation to previous results, the surface hydrophobicity of legumin in SCN^- ($\mu = 1.0$) appeared appropriate for micelle formation.

For the two moderately stabilizing salts, $C_2H_3O_2^-$ and Cl^-, the micelle response decreased to rating 2 at $\mu = 1.0$ (Table 4). Both salts are known to promote predilution preferential hydration of the surface of proteins in general (Arakawa and Timasheff, 1982). For legumin, this phenomenon was reflected by the significant decrease in surface hydrophobicity (S_a and S_c) with increasing $C_2H_3O_2^-$ concentrations. This hydration effect, however, may have been reduced by specific binding of the anions to the protein surface (Arakawa and Timasheff, 1982). As a result, reduced surface hydrophobicity did not allow extensive molecular association. Secondly, post-dilution binding of $C_2H_3O_2^-$ and Cl^- to the protein surface may have created prohibitory electrostatic effects that did not encourage massive association of formed micelles.

Sodium citrate ($Na_3C_6H_5O_7$) as a stabilizing salt caused a decrease in micelle response to a zero rating at $\mu = 1.0$ (Table 4). This was due to a significant decrease in the surface hydrophobicity (both S_c and S_a) with increasing salt concentration, presumably as a result of preferential hydration of the protein surface. Although the aromatic hydrophobicity was still high in relation to other salts, the skewed hydrophobicity balance may still have not been appropriate for micelle formation and interaction.

Effect of urea on micelle formation by legumin

The rationale for the urea experimentation was to investigate micellization in terms of the delicate hydrophilic-hydrophobic balance, with environments of different denaturing capacities. The impact of disrupting noncovalent interactions was monitored in terms of conformational stability and micelle response of legumin as a function of varying levels of urea concentration.

An initial micelle response (rating 5) was observed with legumin exposed to 0.5M urea (Table 5). With an increase in the concentration to 1.0M urea, the micelle response diminished significantly (rating 2). This reaction was also observed with vicilin from *Vicia faba* (Ismond et al., 1988). Conformational disturbances were evident in terms of a significant fluctuation in Td values. Interestingly, the Td values for 0.5, 1.0 and 2.0M urea were higher than the control. Other parameters (△H, S_c, S_a), however, were constant. Although conformational changes appeared minimal, the molecular interactive capacity significantly changed to result in reduced micelle re-

sponse. Sensitivity to low urea concentrations has been reported for other proteins. For example, Yao et al. (1984) observed creatine kinase was inactivated at urea levels that had no influence on protein conformation.

The micelle response deteriorated further as urea level increased from 1.0M (rating 2) to 8.0M (rating 1). This was paralleled by linear decreases in S_c, $S_,$, Td and $\triangle H$ (Table 5). In terms of thermal parameters, a reduction in Td usually correlates with molecular destabilization. Complete denaturation is reflected by low $\triangle H$ values. Privalov and Khechinashvili (1974) stated the denaturation enthalpy for globular proteins was the result of two opposite the thermodynamic effects — an exothermic response resulting from the disruption of hydrophobic associations with denaturation and an endothermic response resulting from dissolution of hydrogen bonds. Any decrease in the $\triangle H$ value reflects a change in original conformation of the protein prior to calorimetric denaturation. The decrease in both aromatic and aliphatic hydrophobicity values was unexpected; deterioration of conformation to a random coil should be paralleled by an increase in exposure of hydrophobic residues. When a globular protein undergoes denaturation, hydrophobic groups located on the inside of the globular structure would be exposed to the outside environment. However, the decreased hydrophobicity values observed here may reflect intermolecular aggregation as denaturation progressed. That is, there may have been a critical decrease in exposed hydrophobic residues as a result of intermolecular aggregation prior to induction of the micelle response. Other studies have shown noncovalent associations of various proteins with denaturant-induced unfolding (Prakash and Nandi, 1977; Fish et al., 1985; Ismond et al., 1988).

In summary, micelle formation and subsequent interaction were influenced by the environment. Firstly, the pH of the surrounding medium had to be low enough to minimize electrostatic repulsion yet high enough to allow protein solubilization. A pH range of 5.5 to 6.5 was appropriate. Secondly the concentration and identity of environmental anions affected micelle formation. At lower salt concentrations (μ = 0.25 and 0.5), the exposure of a specific level of hydrophobic residues appeared critical. Exaggerated hydrophobicity values, as observed with the citrate anion, were detrimental to micelle response. At higher concentrations (μ = 1.0), increased binding of the anion (Cl^-, $C_2H_3O_2^-$, SCN^-) to the protein surface created an electrostatic environment unfavorable to micellization. Thirdly, gradual denaturation of legumin by increasing urea resulted in low hydrophobicity values. Molecular aggregation prior to introduction of an aqueous environment apparently had a negative influence on establishment of micelle-based structures. In all cases, a delicate balance of hydrophilic-hydrophobic noncovalent forces appeared critical to micelle formation and subsequent interaction. These are important considerations if micelle formation is to be used in network formation in various food systems.

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Effect of Total Soymilk Solids on Acid Production by Selected *Lactobacilli*

CHUN-YEN CHANG and MARTHA B. STONE

ABSTRACT

Soymilk was prepared from soybeans using extraction ratios of 1:6, 1:7, 1:8, 1:9, and 1:10 (dry beans:water). Total solids, protein, and carbohydrate increased when extraction ratios of soybeans to water were increased. Soymilk was fermented by *Lactobacillus fermentum* NRRL B-585 or *Lactobacillus acidophilus* NRRL B-1910 or B-2092 and evaluated as a substrate for acid production. Mean values of acid production were: NRRL B-585 7.00 to 16.15, B-1910 32.30 to 39.10, and B-2092 27.20 to 34.85 (μmole lactic acid/gram soymilk). B-1910 and B-2092 grew better than B-585, in soymilk without fortification. Effects of protein and carbohydrate contents of soymilk on acid production were different among strains of lactobacilli.

INTRODUCTION

SOYBEANS are an excellent source of oil and protein. They supply about 80% of the edible vegetable oils used in the U.S., and the meal residue is used as feed for livestock and poultry (Wolf and Cowan, 1975). Because of the increasing gap between worldwide supply and demand for high quality food products, shifts from animal to vegetable sources of protein have increased significantly. Use of soybeans as a direct source of edible protein is more efficient than using them as a feed. However, direct consumption of soybeans has been limited by their flavor and flatus-inducing problems. The undesirable flavor of soybeans is described generally as beany, bitter, grassy, and astringent and is attributed partially to degradation of lipids. Lack of α-galactosidase for complete hydrolysis of carbohydates in soybeans (low molecular weight oligosaccharides including sucrose, raffinose, and stachyose) results in production of gases in the gastrointestinal tract of humans (Gitzelmann and Auricchio, 1965). Some lactic acid bacteria (LAB) that can use sucrose and galacto-oligosaccharides for growth and acid production can be used to manufacture a fermented product from soymilk, a water extract of whole soybeans. This helps remove flatus-inducing sugars (Mital and Steinkraus, 1975). Production of volatile diacetyl and a reasonable level of activity by lactic acid bacteria would also help improve flavor and keeping quality of soymilk (Pinthong et al., 1980).

The level of total solids in cow milk has significant effects on both the consistency and aroma of manufactured yogurt. An increase in total solids results in an increase in titratable acidity and a reduction in coagulation time (Tamime and Deeth, 1980). However, in a study on the effect of total solids on activity of yogurt starters, Pulay and Krasz (1974) concluded that higher than 25% total solids adversely affected availability of moisture and hindered starter activity. In order to make a desirable yogurt-like fermented soymilk, the level of total solids in soymilk must be considered. One easy and inexpensive way to increase the level of total solids in soymilk is to increase the extraction ratio of soybeans to water. The proteins and carbohydrates in the total solids of soymilk are different from those in cow milk. Therefore, different effects of total solids on starter activity were expected in soymilk. The objective of our study was to determine the composition of soymilk when extraction ratios were varied and the effects of composition on growth of LAB and their acid production.

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MATERIALS & METHODS

Preparation of soymilk

Dry, mature, whole, Williams 82 soybeans obtained locally were used. Fifty grams of beans were soaked in tap water overnight at room temperature (22–25°C). The beans were washed, drained, and ground in hot tap water (80–90°C) 3 min in an Osterizer blender (Model Imperial VIII, Milwaukee, WI). Water (300, 350, 400, 450, or 500 mL) to give extraction ratios of 1:6, 1:7, 1:8, 1:9, and 1:10 (dry beans: water, w/v) was poured over beans in the blender. The resulting suspension was filtered through three layers of cheesecloth, dispensed into culture tubes, autoclaved 20 min at 121°C, and held overnight at 5°C.

Composition of soymilk

An AOAC (1984) method was used to determine total solids in soymilk as percentage of residue remaining after drying. Protein content was determined by the AOAC (1984) micro-Kjeldahl procedure, using 6.25 as a nitrogen to protein conversion factor for estimation of protein. Barium hydroxide and zinc sulfate solutions were added to the soymilk to denature proteins before carbohydrate analysis. After centrifugation, the supernatant was analyzed by a colorimetric method (Dubois et al., 1956) to determine total carboydrates.

Cultures

Lactobacillus acidophilus NRRL-2092 and NRRL-1910 and *Lactobacillus fermentum* NRRL B-585 were obtained from the USDA Northern Regional Research Laboratory in Peoria, IL. Cultures were maintained by weekly transfer in Lactobacillus MRS broth and held at 5°C between transfers. Purity of cultures was checked periodically by Gram's staining procedure. All cultures were transferred in soymilk daily for 2 days before they were used to prepare 16–18 hr-old inocula for soymilk fermentation. The incubation temperature was 37°C.

Acid production and changes in pH

Soymilk (25 mL) was inoculated aseptically with 0.5 ml of 16-18 hr-old inoculum at a rate of 2%. Three different strains of lactobacilli and soymilk made with the five different extraction ratios gave 15 treatment combinations. All inoculated soymilks were incubated at 40°C for 16 hr. Acid development was measured by titration of 1g fermented soymilk with 0.02N NaOH, using phenolphthalein as the indicator. Changes in pH also were monitored with a pH meter (Corning Scientific Co., Medfield, MA).

Statistical analysis

The entire experiment was replicated four times. Analysis of variance and Least Significant Difference (LSD) tests were performed to determine differences in treatments. Multiple regression analysis of each factor was conducted to prepare a predictive model for acid production by each strain of lactobacilli. These fitted models were used to develop illustrative three-dimensional response surfaces. All statistical analyses were made with the Statistical Analysis System software package (SAS Inc., 1985).

Table 1 – Composition of soymilk from various bean:water ratios[a]

Extraction Ratio (w/v) Bean:Water	Solids %	Protein %	Carbohydrate %
1:6	9.73[A]	4.85[A]	3.79[A]
1:7	8.66[B]	4.41[B]	3.38[B]
1:8	7.77[C]	3.90[C]	3.09[C]
1:9	6.98[D]	3.61[C]	2.88[C]
1:10	6.40[E]	3.21[D]	2.61[D]

[a] Mean values in same column with different superscripts are significantly different ($p<0.05$, $r=4$).

Table 2 – Final pH values and acid production for fermented soymilk[a]

Extraction ratio (w/v)	NRRL B-585		NRRL B-1910		NRRL B-2092	
Bean:Water	pH	DA[b]	pH	DA	pH	DA
1:6	5.70	16.15[A] (0.15%)	5.00	32.30[A] (0.29%)	4.94	32.35[AB] (0.29%)
1:7	5.79	10.85[AB] (0.10%)	4.81	33.80[A] (0.30%)	4.73	34.85[A] (0.31%)
1:8	5.79	10.25[B] (0.09%)	4.69	36.40[A] (0.33%)	4.68	32.35[AB] (0.29%)
1:9	5.81	9.35[B] (0.08%)	4.64	39.10[B] (0.35%)	4.60	31.25[AB] (0.28%)
1:10	5.88	7.00[B] (0.06%)	4.74	35.65[A] (0.32%)	4.71	27.20[B] (0.24%)

[a] Means of four replicates. Means in same column with different superscripts are significantly different ($p<0.05$).

[b] DA = Developed acidity (reported as μmole lactic acid/g soymilk); percentage of DA as lactic acid (w/w) indicated in parentheses.

RESULTS & DISCUSSION

Composition of soymilk

Total solids in soymilk include protein, carbohydrate, lipid, and ash. Since the effects of lipid and ash were not considered, soymilks were analyzed only for total solids, protein, and carbohydrate (Table 1). Total solids of soymilk increased significantly when extraction ratios of soybeans to water were increased ($P<0.05$). Protein and carbohydrate contents also increased when soybean-to-water ratios were increased, though differences between 1:8 and 1:9 ratios were not significant. Protein and carbohydrate contents correlated positively with total solids content ($p<0.01$). However, the correlation between protein and total solids ($r=0.94$) was higher than the correlation between carbohydrate and total solids ($r=0.72$).

Changes in pH and acid production

pH of autoclaved soymilk was about 6.5. After incubation, acids developed in inoculated soymilk, and pH of soymilk decreased as shown in Table 2. Because of differing capabilities of utilizing oligosaccharides in soymilk, pH decreases were different in soymilk fermented by different lactic acid bacteria.

LAB are Gram-positive, nonspore-forming bacteria that produce lactic acid as the major or sole product of fermentation. The homofermentative LAB produce two molecules of lactic acid from one molecule of glucose. The heterofermentative LAB produce one lactic acid, ethanol, and one carbon dioxide molecule from one molecule of glucose (Fung, 1986). When more substrate is available, more LAB can grow, and, therefore, more acid is produced in the environment. Both homofermentative oganisms, *L. acidophilus* NRRL B-1910 and B-2092, and a heterofermentative type, *L. fermentum* NRRL B-585, were used in our study, and their growth was evaluated in terms of acid production.

The amount of acid produced in soymilk fermented by each strain of lactobacilli is shown in Table 2. Mean values of acid production were: *L. fermentum* NRRL B-585, 7.00 to 16.15; *L. acidophilus* NRRL B-1910, 32.30 to 39.10; and NRRL B-2092, 27.20 to 34.85 (μmole lactic acid/gram fermented soymilk). Apparently, NRRL B-1910 and B-2092 grew better than B-585, in soymilk without fortification.

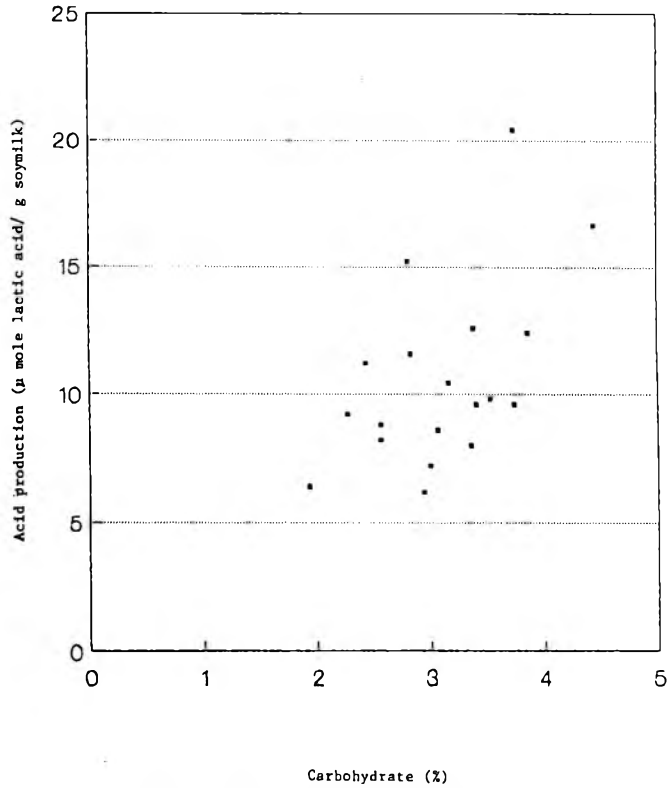

Fig. 1 – Relationship between acid production by L. fermentum NRRL B-585 and carbohydrate content in soymilk

Acid production by lactobacilli varied as the extraction ratio of beans to water or solids content increased (Table 2). *L. fermentum* NRRL B-585 had maximum acid production in soymilk with a 1:6 extraction ratio. *L. acidophilus* NRRL B-2092 produced more acid as the extraction ratio of soymilk was increased from 1:10 to 1:7 and had maximum acid production at 1:7. Acid development than decreased in soymilk with 1:6 extraction ratio. *L. acidophilus* NRRL B-1910 produced the maximum amount of acid in soymilk with 1:9 extraction ratio. Acid production decreased at either higher or lower extraction ratios.

Total solids content higher than the optimal level seemed to inhibit growth of LAB in terms of acid production in soymilk. It was interesting that the activity of LAB was inhibited at such a low total solids level (up to 9.73%) in soymilk, whereas 25% total solids was the level reported to inhibit the activity of LAB in bovine milk (Pulay and Krasz, 1974).

The relationships between acid production and total carbohydrates of soymilk were plotted from raw data (Fig. 1 to 3). Acid production by *L. fermentum* NRRL B-585 increased when carbohydrate content in soymilk increased (Fig. 1). A level of total solids in soymilk as high as 9.73% did not inhibit growth of B-585. This indicated B-585 could grow better and develop more acid in soymilk from extraction ratio higher than 1:6 based on extrapolation of data. However, the total amount of acid from B-585 was much less than from the two other strains. A previous investigation (Mital and Steinkraus, 1975) showed *L. fermentum* B-585 was not able to ferment sucrose, although it could completely utilize raffinose in 12 hr and stachyose in 25 hr. Since sucrose is the major sugar in soybeans, it was not surprising that B-585 could not grow well in soymilk.

Carbohydrate content in soymilk had a quadratic effect on acid production by *L. acidophilus* B-1910 (Fig. 2). Acid production by B-1910 increased as the carbohydrate content in soymilk was increased, until an optimal level was reached. Above this optimal level, acid production decreased when car-

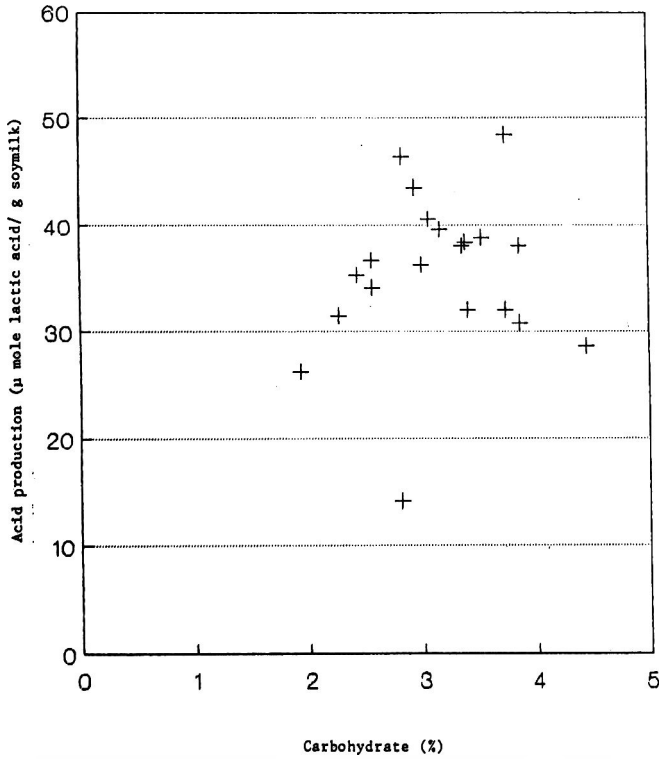

Fig. 2 — Relationship between acid production by L. acidophilus NRRL B-1910 and carbohydrate content in soymilk

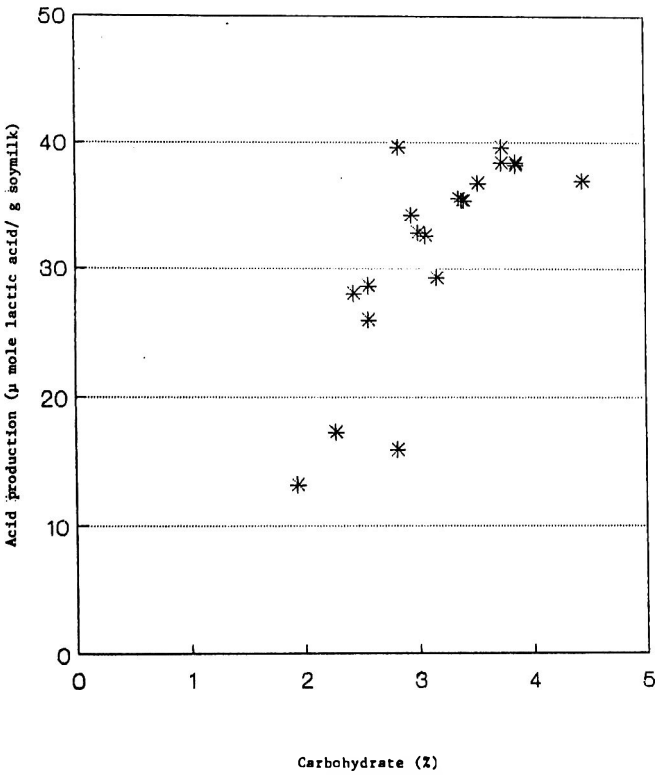

Fig. 3 — Relationship between acid production by L. acidophilus NRRL B-2092 and carbohydrate content in soymilk

bohydrate in soymilk was increased, as though excessive carbohydrate inhibited growth of B-1910. This strain grew relatively well in soymilk based on the amount of acid production (Table 2). Good utilization of oligosaccharides and subsequent accumulation of degraded products from oligosacchrides progressed during fermentation with this organism. The inhibition of lactobacilli activity might be attributed to pH decrease and excessive amounts of galactose and melibiose. Ohtakara et al. (1984) showed that the activity of α-galactosidase, which is present in LAB and is known to hydrolyze α-galactosyl linkages in oligosaccharides, was inhibited greatly by galactose and melibiose.

Acid production by *L. acidophilus* NRRL B-2092 increased when carbohydrate content in soymilk increased (Fig. 3).

Regression analysis

Multiple regression analysis of acid production on solids content (SOL) in soymilk was performed. The quadratic models for these three strains of lactobacilli were as follows:

NRRL B-585:

$$ACID = -29.9428 + 8.1969\ SOL - 0.3770\ SOL^2$$

$$R^2 = 0.5599$$

NRRL B-1910:

$$ACID = 65.5123 - 6.4139\ SOL + 0.3223\ SOL^2$$

$$R^2 = 0.0419$$

NRRL B-2092:

$$ACID = -27.3489 + 12.6089\ SOL - 0.6360\ SOL^2$$

$$R^2 = 0.1361$$

Quadratic models for B-1910 and B-2092 based on solids contents were inadequate predictors for acid production. Using protein (PRT) and carbohydrate (CAR) contents as regressors improved the estimation of acid production by B-1910 and B-2092. Least squares equations containing quadratic and cross-product terms were:

NRRL B-585:

$$ACID = -20.0233 + 9.1474\ PRT + 2.1065\ CAR - 0.2840\ PRT^2 + 0.5773\ CAR^2 - 1.0767\ PRT \times CAR$$

$$R^2 = 0.5952$$

NRRL B-1910:

$$ACID = 25.1734 + 0.8861\ PRT + 10.5233\ CAR - 9.2706\ PRT^2 - 15.9689\ CAR^2 + 22.6781\ PRT \times CAR$$

$$R^2 = 0.5738$$

NRRL B-2092:

$$ACID = -36.0846 + 6.8468\ PRT + 29.1433\ CAR - 5.5145\ PRT^2 - 10.2592\ CAR^2 + 11.2994\ PRT \times CAR$$

$$R^2 = 0.8202$$

A reverse elimination procedure was employed in an attempt to remove all unnecessary terms from the full models without substantially increasing the size of the estimate of model variance. In this way, the best fitted models were selected (Draper and Smith, 1981).

NRRL B-585:

$$ACID = -5.9514 + 4.1690\ PRT$$

$$R^2 = 0.5499$$

NRRL B-1910:

All variables were retained in the model.

NRRL B-2092:

$$ACID = -34.6686 - 3.4052\ PRT + 40.1261\ CAR - 4.5171\ CAR^2$$

$$R^2 = 0.7543$$

Three-dimensional distributions of predictive acid production by three strains of lactobacilli are shown in Fig. 4 to 6, when carbohydrate (2.00 – 4.50%) and protein (3.00 – 5.00%) contents of soymilk were varied. To check fitness of these models,

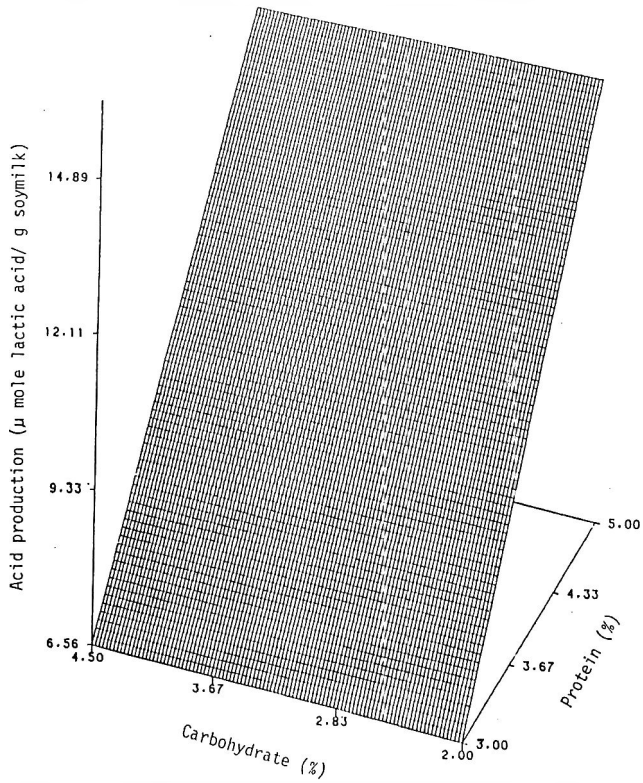

Fig. 4 — Predictive acid production by L. fermentum B-585 as a function of protein and carbohydrate contents in soymilk

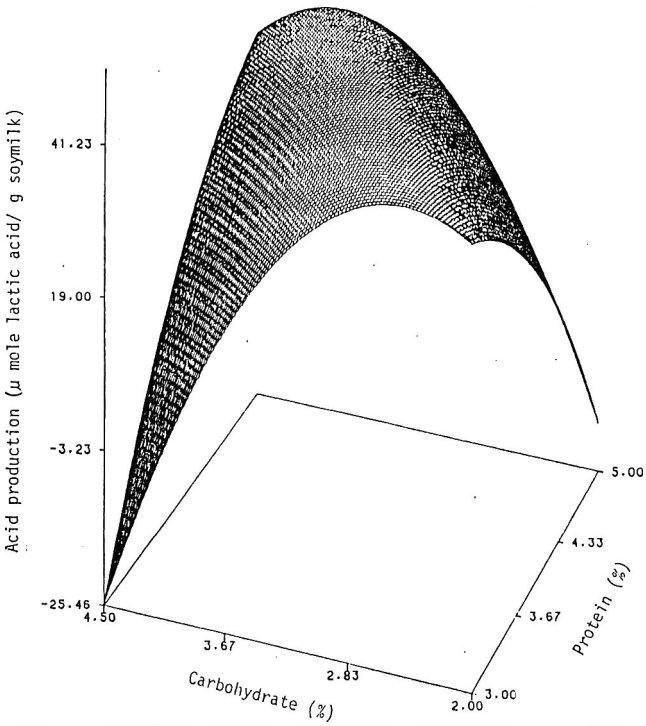

Fig. 5 — Predictive acid production by L. acidophilus B-1910 as a function of protein and carbohydrate contents in soymilk

protein and carbohydrate data from Table 1 were substituted into the equations to calculate predictive acid production. Some of these predictive values were close to experimental observations (Table 2), but some were not. This indicated factors other than protein and carbohydrate in soymilk should be included in the model for better estimation, especially when NRRL B-585 and B-1910 were the fermentation organisms.

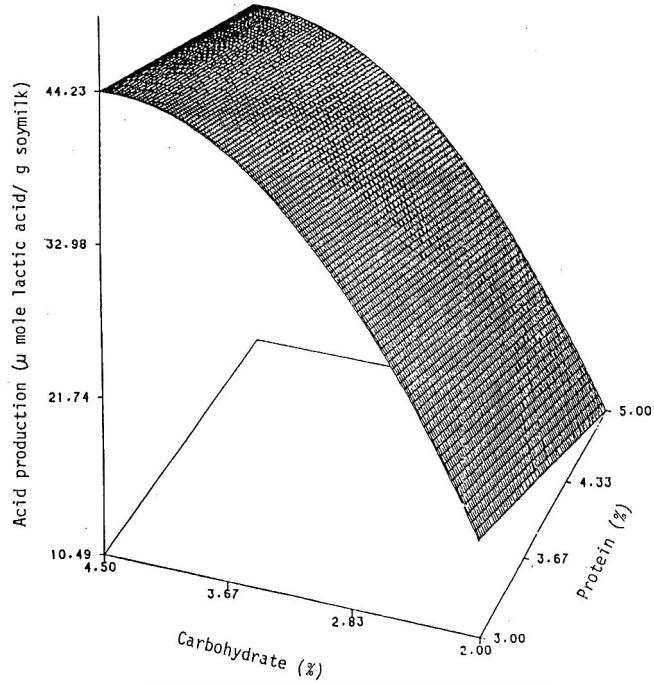

Fig. 6 — Predictive aid production by L. acidophilus B-2092 as a function of protein and carbohydrate contents in soymilk

CONCLUSIONS

TO MAKE acceptable fermented soymilk, the consistency and acid development of the final product were critical. Use of soymilk with reasonable amounts of fermentable carbohydrates and protein, was necessary so lactobacilli could develop enough acid to produce a soft curd from soy protein. *L. acidophilus* NRRL B-1910 and B-2092 grew well and produced sufficient acid to form a yogurt-like product at five different extraction ratios. *L. fermentum* NRRL B-585 was not a good starter culture for fermented soymilk because of its poor utilization of sucrose.

In yogurt making, total solids of bovine milk is usually the only factor considered. However, when soymilk is used as a substrate for growth of lactic cultures, factors other than total solids, such as protein and carbohydrate, should be considered. Because each strain of lactobacilli showed a different pattern of growth in soymilk, adjustment of protein and carbohydrate contents in soymilk was necessary to optimize growth of a specific strain.

Regression models including protein and carbohydrate content as variables were not adequate to predict acid production by lactobacilli. Lactic acid fermention is a complicated biochemical process and other factors that influence fermentation, such as incubation temperature, and time, and formation of degradation products, should be studied and possibly included in the model.

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Effect of Heat Treatments on Chemical Analysis of Dietary Fiber

M.-C. CHANG, and W. C. MORRIS

ABSTRACT

Apple fiber, corn fiber, oat bran and soy fiber were analyzed to study the effect of heat processing (autoclaving at 121°C/15 min, 100°C/30 min and microwave heating for 5/10 min) on dietary fiber fractions. Samples were analyzed for insoluble (IDF), soluble (SDF), and total dietary fiber (TDF) by an enzymatic-gravimetric method. Autoclaving reduced IDF of apple fiber and TDF of apple fiber and oat bran. Microwave heating reduced TDF in apple fiber and oat bran and IDF in oat bran but increased the SDF of apple fiber. All treatments decreased the SDF in corn fiber. Effects on dietary fiber fractions depended on fiber type and processing method.

INTRODUCTION

DIETARY FIBER is derived from various tissues of fruits, vegetables, cereals, and legumes; therefore, general conclusions are not easy to derive about the physicochemical characteristics of dietary fiber. Fiber-supplemented foods have been formulated by using dietary fiber from fruits, woods, cereals and legumes to manufacture high fiber ingredients which are then used in various food systems. Normally, dietary fiber is subjected to heat processing such as pressure cooking, baking, frying, microwave heating or extrusion cooking prior to consumption.

The Federation of American Societies of Experimental Biology has recommended daily consumption of 20 to 35 grams of dietary fiber from various fruits, whole grains and legumes (Andres, 1987). Growing consumer awareness of the nutritional attributes of dietary fiber has led to an increased demand for high fiber products. Consequently, more raw fiber is being incorporated into processed foods as a functional ingredient; however, manufacturing and processing conditions or home preparation may affect chemical composition of the fiber which in turn may alter its physiological role in the human body (Payne, 1987; Weber and Chaudhary, 1987). Research has been conducted on various functional properties of fiber, including substitution, water-holding capacity and oil-holding capacity (Babcock, 1987; Childs and Abajian, 1976; Collins and Post, 1981; Polizzoto et al., 1983). Many studies have compared the relationship of the physiological effects to the content and chemical composition of dietary fiber (Anderson, 1985; Anderson et al., 1987; Cummings, 1987; Mueller et al., 1983; Rasper, 1979; Selvendran, 1984). Few studies have investigated the effect of processing techniques or heat treatments on dietary fiber and the insoluble/soluble fiber ratio in foods.

The effect of cooking on fiber content of foods is still unclear (Asp et al., 1982; Matthee and Appledorf, 1978; Varo et al., 1984; Zyren et al., 1983). Since some browning products are analyzed as lignin, cooking that causes browning reactions can increase the apparent fiber content of the food. Canned vegetables may appear to have higher fiber contents than fresh vegetables because browning reactions may occur with cooking during thermal processing. Also, water may be lost from the vegetables during processing of the canned product resulting in increased dietary fiber. Englyst et al. (1982, 1983) proposed that digestion resistant starch is produced as a result of subjecting foods to heat or dehydration processes, conferring more ordered structures on starch molecules and making them less amenable to enzyme digestion. If a food component is not digested, then it would be considered part of dietary fiber. Bjorck et al. (1984) studied the effect of extrusion cooking on wheat fiber and found that raw wheat flour had 40% soluble dietary fiber (SDF) while the extruded flour had 50 to 75% SDF. Thermal processing made a small amount of the starch less available to enzymes and thus increased the dietary fiber value (Bjorck et al., 1984; Varo et al., 1983). Jones et al. (1985) found the amount of starch resistant to *in vitro* hydrolysis by enzymes increased by 30 to 50% in cooked versus raw potatoes. Autoclaving tended to reduce total dietary fiber (TDF) content, while baking decreased the TDF in tomatoes (Varo et al., 1984).

Numerous researchers have studied the chemical, nutritional, and epidemiological nature of dietary fiber, while few studies have investigated the effect of processing on the various fractions of dietary fiber. The fiber content of foods or food products has historically been determined by the crude fiber procedure (AOAC, 1984). Only part of the dietary fiber (cellulose and lignin) is measured in this procedure. TDF consists of nonstarch polysaccharides and lignin with smaller amounts of minerals, plant lipids, protein and other substances. The objective of our research was to determine what effect further heat processing treatments may have on the insoluble dietary fiber (IDF), SDF and TDF in apple fiber, corn fiber, oat bran and soy fiber.

MATERIALS & METHODS

Experimental design

The following types of fiber were analyzed in this study: apple fiber (Tastee Apple, Inc., Newcomerstown, OH), dry milled corn fiber (Illinois Cereal Mills, Inc., Paris, IL), oat bran (The Quaker Oats Co., Chicago, IL), and soy fiber (Hi-Pro F300, Grain Processing Co., Muscatine, IA). The processing conditions were autoclaving, which simulated general processing conditions (121°C, 15 min) and general cooking conditions (100°C, 30 min) and microwave heating (700 Watts, 2450 MHz) for 5 and 10 min (Schrumpf and Charley, 1975; Varo et al., 1984). Preliminary studies indicated the fiber could not be directly microwave-heated for extended periods without severe burning. Consequently, the optimum ratio of water-to-fiber was determined by adding enough water to assure all dry materials were wetted. This mixture was well mixed for the microwave heat process. The optimum ratios (w/w) were: apple 4.5:1, corn 3:1, oat 3:1, soy 9:1.

The statistical design was completely randomized with each process duplicated and each product chosen randomly and analyzed in duplicate.

Proximate analyses

Ash was determined by ignition in a muffle furnace; moisture was analyzed by drying in an air oven according to the AOAC (1984) procedure. Samples previously dried for moisture determination were weighed into an ether extraction thimble to determine the crude fat (AOAC, 1984). Protein was obtained using the Kjeldahl method (AOAC, 1984). The percentage crude protein was calculated as Nx6.25.

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Table 1—Proximate composition[a], total dietary fiber[b], and smoking time of unprocessed apple fiber, corn fiber, oat bran and soy fiber

Product	Ash (%)	Crude fat (%)	Moisture (%)	Crude protein (%)	Total dietary fiber (%)	Smoking time (min)
Apple fiber	1.91 ± 0.03	4.14 ± 0.18	6.07 ± 0.12	4.11 ± 0.16	51.01 ± 4.02	2
Corn fiber	0.91 ± 0.04	2.51 ± 0.20	8.39 ± 0.11	3.55 ± 0.19	78.47 ± 0.78	7
Oat bran	3.55 ± 0.20	5.17 ± 0.08	9.78 ± 0.11	17.33 ± 0.18	22.02 ± 1.80	5.5
Soy fiber	3.63 ± 0.22	1.73 ± 0.08	10.87 ± 0.07	21.38 ± 0.16	62.26 ± 1.65	8.5

[a] Mean ± S.D. of 4 determinations; wet weight basis.
[b] Mean ± S.D. of 4 determinations; dry weight basis.

Table 2—Effect of heat treatments on percent insoluble dietary fiber in apple fiber, corn fiber, oat bran and soy fiber

		Autoclaved		Microwave heated	
Product	Control	121°C 15 min	100°C 30 min	5 min	10 min
Apple fiber	39.17 ± 1.29[a]	36.00 ± 2.51[b]	37.23 ± 1.18[ab]	36.90 ± 0.31[ab]	36.36 ± 0.13[ab]
Corn fiber	78.25 ± 1.40[a]	79.15 ± 1.12[a]	77.93 ± 0.77[a]	77.78 ± 0.14[a]	79.81 ± 4.06[a]
Oat bran	13.10 ± 2.23[a]	10.18 ± 2.06[a]	9.89 ± 1.10[ab]	9.38 ± 0.91[b]	8.89 ± 0.54[b]
Soy fiber	53.15 ± 4.07[a]	56.82 ± 2.18[a]	57.79 ± 0.73[a]	57.73 ± 3.97[a]	57.78 ± 4.48[a]

[ab] Mean ± S.D. of 4 determinations, on dry weight of product. Means within the same row with different superscripts are significantly different by Tukey's test at 5% significance level.

Table 3—Comparison of means[a] of heat treatments on percent insoluble dietary fiber of apple fiber, corn fiber, oat bran and soy fiber

Product	Control[b] vs all treatments[c]	Control[b] vs autoclaved[d]	Control[b] vs microwave heated[e]	Autoclaved[d] vs microwave heated[e]
Apple fiber	39.17:36.62**	39.17:36.61**	39.17:36.63**	36.61:36.63
Corn fiber	78.25:78.67	78.25:78.54	78.25:78.79	78.54:78.79
Oat bran	13.10:9.58**	13.10:10.04**	13.10:9.13***	10.04:9.13
Soy fiber	53.15:57.53*	53.15:57.30	53.15:57.75*	57.30:57.75

[a] Percent insoluble dietary fiber, on dry weight of product.
[b] n = 4.
[c] n = 16; represents combination of both microwave heat and autoclave treatments.
[d] n = 8; represents combination of both autoclave treatments.
[e] n = 8; represents combination of both microwave heat treatments.
* Significant at $p < 0.05$ level; **Significant at $p < 0.01$ level; ***Significant at $p < 0.001$ level.

Smoke time was the treatment time for the unprocessed fiber sample, with no water added, to generate smoke in the microwave oven set at 700 Watts, 2450 MHz.

Analysis of insoluble, soluble and total dietary fiber

All samples after treatment were vacuum dried to constant weight at 70°C, 12 hrs, 16 psi (Precision Scientific Co., Chicago, IL) before further analyses. Each sample was ground (Wiley Mill, Emerson Electric, St. Louis, MO) through a screen of #40 mesh to ensure homogeneity and uniform particle size. Ground samples were stored in capped plastic containers in a desiccator at ambient temperature for further analysis for dietary fiber. IDF, SDF and TDF were determined by the modified enzymatic-gravimetric method described by Prosky et al. (1988).

Statistical analysis

The processes were replicated twice, and all analyses were in duplicate. Analysis of variance was used to determine significant differences that could be attributed to processing conditions. Tukey's pairwise comparison procedure was performed to note differences among means; differences were estimated between control vs all treatments, control vs autoclaved, control vs microwave heated, and autoclaved vs microwave heated products. These analyses were performed with the Statistical Analysis System (SAS, 1985).

RESULTS & DISCUSSION

Proximate analyses

The proximate analysis of each unprocessed fiber is presented in Table 1. Corn fiber contained higher TDF (79.08%) and lower ash (0.91%) than soy fiber, apple fiber or oat bran. Oat bran and soy fiber were higher in protein and ash than apple or corn fibers. Soy fibers contained more moisture which allowed microwave heat treatment for a longer period before smoking; whereas, apple fiber, with a lower moisture level, had a shorter smoking time. The crude fat in of all samples was less than 10%, thus fat extraction was not required before milling.

Insoluble dietary fiber

The differences between IDF of apple, corn, oat, and soy fibers are shown in Table 2. Autoclaving at 121°C, 15 min significantly decreased IDF content of the apple fiber. Microwave heat treatment 5 and 10 min significantly reduced IDF of oat bran. This effect of heat on IDF was similar to that previously reported in potatoes, carrots and broccoli (Hughes et al., 1975; Ooraikul, et al., 1974; Schrumpf and Charley, 1975). Due to the higher protein in soy, thermal processing may have caused Maillard reaction products which would increase the IDF and TDF values. During fiber analysis of all products the processed sample solutions were ably darker or deeper in color than unprocessed sample solutions. No significant differences caused by processing conditions were observed for the insoluble fraction of corn or soy fiber.

Comparing all heat treatments to the control, the IDF of apple fiber ($p<0.01$) and oat bran ($p<0.001$), were reduced but the IDF of soy fiber ($p<0.05$) increased (Table 3). Autoclaving caused a reduction ($p<0.01$) in the insoluble fraction of apple fiber and oat bran when compared to the control. Autoclaving caused no significant differences in the insoluble fraction of corn or soy fibers when compared to the control. When the microwave processed were compared to the control, the insoluble fraction of apple fiber ($p<0.01$) and oat bran ($p<0.001$) decreased, but in the insoluble fraction of soy fiber increased ($p<0.05$). No differences were observed between the two processing methods in the insoluble fraction of any samples.

Further processing of the insoluble fraction of these four fiber sources indicated heat treatment was most effective in decreasing the insoluble fraction in those fibers that were lowest in the insoluble component (apple 39.17%, oat 13.10%).

Table 4—Effect of heat treatments on percent soluble dietary fiber in apple fiber, corn fiber, oat bran and soy fiber

Product	Control	Autoclaved		Microwave heated	
		121°C 15 min	100°C 30 min	5 min	10 min
Apple fiber	9.38 ± 0.79[a]	12.11 ± 2.21[ab]	11.38 ± 0.36[ab]	13.37 ± 1.39[b]	13.38 ± 1.76[b]
Corn fiber	0.70 ± 0.12[a]	0.21 ± 0.20[b]	0.15 ± 0.14[b]	0.24 ± 0.04[b]	0.18 ± 0.19[b]
Oat bran	5.64 ± 1.74[a]	4.45 ± 1.21[a]	6.17 ± 0.44[a]	5.55 ± 2.17[a]	4.63 ± 2.02[a]
Soy fiber	7.35 ± 1.38[a]	6.88 ± 0.51[a]	6.63 ± 0.25[a]	6.91 ± 0.36[a]	7.16 ± 0.19[a]

[ab] Mean ± S.D. of 4 determinations, on dry weight of product. Means within the same row with different superscripts are significantly different by Tukey's test at 5% significance level.

Table 5—Comparison of means[a] of heat treatments on percent soluble dietary fiber of apole fiber, corn fiber, oat bran and soy fiber

Product	Control[b] vs all treatments[c]	Control[b] vs autoclaved[d]	Control[b] vs microwave heated[e]	Autoclaved[d] vs microwave heated[e]
Apple fiber	9.38:12.56**	9.38:11.75*	9.38:13.37***	11.75:13.37*
Corn fiber	0.70:0.19***	0.70:0.18***	0.70:0.21***	0.18:0.21
Oat bran	5.64:5.20	5.64:5.31	5.64:5.09	5.31:5.09
Soy fiber	7.35:6.89	7.35:6.75	7.35:7.03	6.75:7.03

[a] Percent soluble dietary fiber, on dry weight of product.
[b] n = 4.
[c] n = 16; represents combination of both microwave heat and autoclave treatments.
[d] n = 8; represents combination of both autoclave treatments.
[e] n = 8; represents combination of both microwave heat treatments.
* Significant at p < 0.05 level; **Significant at p < 0.01 level; ***Significant at p < 0.001 level.

Table 6—Effect of heat treatments on percent total dietary fiber in apple fiber, corn fiber, oat bran and soy fiber

Product	Control	Autoclaved		Microwave heated	
		121°C 15 min	100°C 30 min	5 min	10 min
Apple fiber	51.01 ± 4.02[a]	47.76 ± 1.90[ab]	46.71 ± 0.13[b]	47.70 ± 1.35[b]	45.78 ± 0.77[b]
Corn fiber	78.47 ± 0.78[abc]	77.00 ± 1.39[bc]	76.51 ± 0.89[c]	80.19 ± 2.27[a]	79.70 ± 3.74[ab]
Oat bran	22.02 ± 1.80[a]	17.34 ± 3.42[b]	19.24 ± 0.88[ab]	16.57 ± 0.38[b]	16.97 ± 0.70[b]
Soy fiber	62.26 ± 1.65[a]	63.54 ± 1.00[a]	63.79 ± 0.78[a]	64.29 ± 1.20[a]	64.02 ± 1.28[a]

[abc] Mean ± S.D. of 4 determinations, on dry weight of product. Means within the same row with different superscripts are significantly different by Tukey's test at 5% significance level.

When the insoluble fraction made up more than half of the fiber source (corn 78.25%, soy 53.15%), there was no significant change of the insoluble fraction when the fiber was heat treated; however, the insoluble component is soy fiber tended to increase with further processing. This may be due to increased Maillard reaction products. Thus, the effects of heat treatment appeared to depend on the fiber source and possibly on the relative quantities of IDF.

Soluble dietary fiber

The effect of the heat treatments on SDF are presented in Table 4. Microwave heat treatment for 5 and 10 min produced significant increases in the SDF of the apple fiber. All heat treatments resulted in significant reduction of the SDF in corn; however, it should be noted that the original soluble fraction in corn was very low. Varo et al. (1984) concluded that autoclaving may hydrolyze some of the water soluble components and decrease the soluble fraction in tomatoes. Our data showed no significant change in the SDF of oat bran or soy fiber during processing. Zyren et al. (1983) made a similar conclusion about processed apple, carrots, lima beans, peas, spinach and sweet potatoes.

Comparison of the soluble fraction in heat treated samples to the control samples showed it increased ($p<0.001$) in the apple fiber, reduced ($p<0.001$) in the corn fiber and did not change in the oat bran or soy fiber (Table 5). When comparing the autoclave and the microwave heat treated samples to controls, SDF increased in apple fiber, was reduced in corn fiber and did not change in oat bran and soy fiber. The microwave heat treatment tended to increase the SDF content of the apple fiber more than did the autoclave treatment.

These results illustrated the effects of different processing conditions on SDF components from different products. The soluble fraction in apple and corn samples was significantly affected by further processing, being increased in the apple fiber and decreased in the corn fiber. The soluble fraction in oat bran and soy fiber was not significantly affected by further processing. Thus, the effect of processing on the SDF fraction also appeared to depend on the fiber source and the processing method.

Total dietary fiber

The effects of processing on the TDF are shown in Table 6. TDF of apple fiber and oat bran decreased whereas, there was no difference in TDF of the soy fiber. Autoclaving at 100°C for 30 min, and treating in the microwave oven for 5 and 10 min caused a significant reduction in the TDF content of apple fiber. Autoclaving at 121°C, 15 min and microwave heat treatment at 5 and 10 min yielded a significant reduction in TDF of oat bran. No heat treatments produced any difference in the TDF content of corn or soy fiber.

When comparing all heat treatments with the control, TDF of apple and oat was reduced ($p<0.0001$); whereas, that of soy fiber increased ($p<0.05$), and corn fiber TDF showed no change (Table 7). A reduction in the TDF occurred in apple fiber ($p<0.001$), corn fiber ($p<0.05$), and oat bran ($p<0.01$) when autoclaved. Results, comparing microwave heated samples to control samples, illustrated how TDF decreased ($p<0.001$) in apple fiber and oat bran but increased ($p<0.05$) in soy fiber. When comparing samples autoclaved to those microwave heated, no difference was observed in the TDF of apple, oat and soy fibers, but a difference ($p<0.001$) was seen in corn fiber (Table 7). The results indicated different processing methods resulted in different effects on TDF when analyzed by our enzymatic-gravimetric method.

Crude protein

Results of processing treatments on the protein content are shown in Table 8. There was a slight, but significant reduction

Table 7—Comparison of means[a] of heat treatments on percent total dietary fiber of apple fiber, corn fiber, oat bran and soy fiber

Product	Control[b] vs all treatments[c]	Control[b] vs autoclaved[d]	Control[b] vs microwave heated[e]	Autoclaved[d] vs microwave heated[e]
Apple fiber	51.01:46.99***	51.01:47.24***	51.01:46.74***	47.24:46.74
Corn fiber	78.47:78.35	78.47:76.75*	78.47:79.94	76.75:79.94***
Oat bran	22.02:17.53***	22.02:18.29**	2.02:16.77***	18.29:16.77
Soy fiber	62.26:63.91*	62.26:63.66	62.26:64.15*	63.66:64.15

[a] Percent total dietary fiber, on dry weight of product.
[b] n = 4.
[c] n = 16; represents combination of both microwave heat and autoclave treatments.
[d] n = 8; represents combination of both autoclave treatments.
[e] n = 8; represents combination of both microwave heat treatments.
* Significant at p < 0.05 level; **Significant at p < 0.01 level; ***Significant at p < 0.001 level.

Table 8—Effect of heat treatments on percent crude protein in apple fiber, corn fiber, oat bran and soy fiber

		Autoclaved		Microwave heated	
Product	Control	121°C 15 min	100°C 30 min	5 min	10 min
Apple fiber	4.27 ± 0.17[ab]	4.35 ± 0.11[a]	4.23 ± 0.03[ab]	4.14 ± 0.09[ab]	4.07 ± 0.09[b]
Corn fiber	3.78 ± 0.16[a]	3.89 ± 0.07[a]	3.85 ± 0.15[a]	3.85 ± 0.02[a]	3.98 ± 0.18[a]
Oat bran	19.24 ± 0.17[a]	19.16 ± 0.47[a]	19.34 ± 0.35[a]	19.55 ± 0.12[a]	19.62 ± 0.11[a]
Soy fiber	23.97 ± 0.17[a]	23.53 ± 0.30[ab]	23.46 ± 0.46[ab]	23.42 ± 0.15[ab]	23.26 ± 0.38[b]

[ab] Mean ± S.D. of 4 determinations, on dry weight of product. Means within the same row with different superscripts are significantly different by Tukey's test at 5% significance level.

Table 9—Comparison of means[a] of percent heat treatments on crude protein of apple fiber, corn fiber, oat bran and soy fiber

Product	Control[b] vs all treatments[c]	Control[b] vs autoclaved[d]	Control[b] vs microwave heated[e]	Autoclaved[d] vs microwave heated[e]
Apple fiber	4.27:4.20	4.27:4.24	4.27:4.15	4.24:4.15
Corn fiber	3.78:3.89	3.78:3.87	3.78:3.91	3.87:3.91
Oat bran	19.24:19.42	19.24:19.25	19.24:19.58	19.25:19.58*
Soy fiber	23.97:23.42**	23.97:23.49*	23.97:23.34**	23.49:23.34

[a] Percent crude protein, on dry weight of product.
[b] n = 4.
[c] n = 16; represents combination of both microwave heat and autoclave treatments.
[d] n = 8; represents combination of both autoclave treatments.
[e] n = 8; represents combination of both microwave heat treatments.
* Significant at p < 0.05 level; **Significant at p < 0.01 level.

in protein content of the soy fiber processed by microwave for 10 min when compared to the control. Autoclaving and microwave heat caused no reduction in protein content of oat bran; however, there was a difference ($p<0.05$) between the two types of processing (Table 9). Autoclaving ($p<0.05$) and microwave heating ($p<0.01$) resulted in a decrease in protein of soy fiber. This change may have been greater due to the higher original protein content of the soy fiber. The fact that the IDF of soy fiber increased supported the hypothesis that processing treatments increased the Maillard reaction products, which were in turn measured as part of the IDF. No difference was observed in protein content of apple, corn, or soy fiber between the two different processing methods.

CONCLUSIONS

THE RESULTS indicate that given processing treatments have different effects on the IDF, SDF, and TDF content of fiber from different sources. This research also shows processing conditions affected the TDF and the insoluble/soluble ratio. The decrease in the TDF of apple and oat fiber was a result of the decreased IDF. The TDF in apple fiber was lower after processing; however, the SDF increased. The decrease in TDF was primarily due to the large decrease in the insoluble fraction. This change in the IDF content would explain the observed changes in the TDF of the processed apple, oat, and soy fiber. We note that in the highest protein-containing fiber, soy, the protein content decreased and the IDF level increased during processing. This supports our theory that, with the fiber method used in our work, we may be measuring an increased level of Maillard reaction products as part of dietary fiber. To further evaluate the effect of processing different treatments under various conditions are needed. Individual chemical components of the fiber fraction need to be analyzed in fibers that have been processed differently.

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High Temperature-Short Time Pasta, Processing: Effect of Formulation on Extrudate Properties

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ABSTRACT

Effects of wheat type and functional ingredients on selected characteristics of pregelatinized pasta manufactured by high temperature-short time twin-screw extrusion were evaluated by physicochemical methods. Although wheat type exerted minor influence on maximum force, total organic matter, and water absorption index, both durum and hard wheats were suitable as raw materials for production of pregelatinized pasta. Most differences in quality characteristics of pasta supplemented with additives were attributable to effects glyceryl monostearate. Neither disodium phosphate (1.0% nor wheat gluten (5.0%) substantially affected physicochemical properties related to textural quality.

INTRODUCTION

IN RECENT YEARS, pasta has become a major growth segment of the cereal foods industry. Although spaghetti, macaroni, and noodles continue to dominate the U.S. market, consumption of novel pasta products has been rising substantially. Innovations in extrusion processing technology have permitted new opportunities for development of convenience foods such as "instant" and "quick-cooking" pasta products that rehydrate rapidly without need for traditional cooking. Recent commercialization of a low-cost, energy-efficient, high temperature-short time (HTST) twin-screw extrusion process has facilitated industrial development of pregelatinized pasta products (Wenger and Huber, 1986). Although Linko et al. (1981) have written a comprehensive review concerning application of HTST extrusion processing to cereal products, specific information concerning production of instant and quick-cooking pasta with such systems is limited.

Several other production techniques have been proposed for manufacture of instant and quick-cooking pasta and noodle products. With a laboratory cooking extruder, Tsao et al. (1976) developed an "instant" spaghetti formulated with commercial white rice meal; however, cooking time in boiling water to reach optimal textural characteristics exceeded 9.0 min. According to Hoskins (1970), the U.S. Army developed a rapid drying method that used radiant heat and vibrating conveyors to create a quick-cooking pasta for military rations, but the process was never commercialized. European and Asian processes for pregelatinized pasta and instant noodles have been engineered such that conventionally extruded or sheeted materials are exposed directly to boiling water, steam, or microwave energy to cook products completely prior to dehydration or deep-fat frying (Cantarelli, 1986; Pagani, 1986; Papotto and Zorn, 1986; Quaglia, 1988).

In the U.S., federal regulations permit either durum wheat or common wheat or their blends to be incorporated into pasta (FDA, 1986). A number of investigators demonstrated that conventionally manufactured spaghetti processed from hard wheat was inferior in quality to similar products from durum wheat (Dexter et al., 1981; Kaci, 1982; Kim, 1987; Kim et al., 1986; Maneepun, 1978; Mousa et al., 1984; Sheu et al., 1967; Tejada, 1982; Wyland and D'Appolonia, 1982). A dull, gray appearance and soft texture of finished product frequently were cited as limiting factors for inclusion of hard wheat in spaghetti formulations. Occasionally, firmness scores for conventional spaghetti products formulated with hard red wheats have exceeded values for products from durum wheats (Kaci, 1982; Sheu et al., 1967; Wyland and D'Appolonia, 1982). However, unlike pasta products from common wheats, spaghetti containing durum wheats consistently exhibited increased resistance to disintegration during prolonged cooking (Dexter et al., 1981; Kaci, 1982; Kim, 1987).

According to federal specifications, functional ingredients currently permitted in macaroni and noodle products are sodium chloride, disodium phosphate, egg albumen, wheat gluten, glyceryl monostearate, and carrageenan (FDA, 1986). Effects of formulation changes on quality characteristics of pregelatinized pasta and noodle products currently are unknown because availability of reports on these products is limited.

Several workers (Kim, 1987; Matsuo et al., 1986; Thoren, 1972) concurred that addition of monoglycerides to conventional pasta enhances cooking quality by preventing stickiness and by diminishing effects of overcooking. Improvements have been attributed to the ability of monoglycerides to form complexes with amylose that delayed onset of gelatinization and prevented migration of amylose from starch granules during heating. Monoglycerides and free fatty acids form similar inclusion compounds with the amylose fraction of starch during twin-screw extrusion cooking (Mercier et al., 1980). Thus, these compounds might provide a beneficial influence on textural quality of pregelatinized pasta products manufactured with HTST extrusion technology.

When vital wheat gluten was added to spaghetti formulations at 3%, improvements in firmness and surface characteristics were noted in comparison to a control (Kim, 1987). Different forms of wheat gluten (flash-dried, spray-dried, activated) incorporated into the formulations produced equivalent changes in cooking properties. Similarly, Breen et al. (1977) found that wheat gluten was a useful adjunct to spaghetti formulations for reduction of cooking losses and improvement in firmness of the cooked product.

Although the influence of sodium or calcium phosphates on cooking characteristics of pregelatinized pasta products has not been reported, FDA (1986) indicated that disodium phosphate reduces cooking time of conventional pasta products; this ingredient could have the potential for acceleration of rehydration rate of pregelatinized pasta as well. Perhaps inclusion of selected functional additives like glyceryl monostearate, wheat gluten, or disodium phosphate in instant and quick-cooking pasta products might permit manufacture of satisfactory products from common wheat materials.

Primary objectives of our investigation were: (1) to ascertain the influence of wheat variety and granulation, together with specific additives (disodium phosphate, wheat gluten, glyceryl monostearate), on selected physicochemical properties of pregelatinized pasta manufactured by HTST extrusion and (2) to establish approximate levels of specific ingredients for addition to such pregelatinized pasta in subsequent studies.

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Table 1—Fractional factorial treatment design for formulations of pregelatinized pasta

Treatment no.[a]	Wheat type	Disodium phosphate[b] (%)	Glyceryl monostearate[c] (%)	Wheat gluten[d] (%)
101	Durum[e]	0	0.75	0
102	Farina[f]	0	0.75	0
103	Semolina[e]	0	0.75	0
104	HWF[g]	0	0.75	0
105	Semolina[e]	1.0	2.0	5.0
106	Semolina[e]	1.0	0.75	0
107	Farina[f]	1.0	0.75	0
108	HWF[g]	0	2.0	5.0
109	Durum[e]	0	2.0	5.0
110	Farina[f]	1.0	2.0	5.0
201	Durum[e]	1.0	2.0	0
202	Durum[e]	1.0	0.75	5.0
203	Semolina[e]	0	0.75	5.0
204	Semolina[e]	0	2.0	0
205	HWF[g]	1.0	2.0	0
206	Farina[f]	0	2.0	0
207	HWF[g]	1.0	0.75	5.0
208	Farina[f]	0	0.75	5.0

[a] Treatments 101-110 were produced on the first day and 201-208 were produced on the second day; 102 and 103 were supplementary formulations processed in addition to the basic fractional factorial design consisting of a one-half replicate of 16 treatments (Cochran and Cox, 1957).
[b] Olin Chemicals, Stamford, CT.
[c] Myvaplex 600®, Eastman Chemical Products, Inc., Kingsport, TN.
[d] Modified vital wheat gluten, Kyowa Hakko Kogyo Co., Ltd., New York, NY.
[e] North Dakota Mill and Elevator, Grand Forks, ND.
[f] Midwest Grain Products, Inc., Atchison, KS.
[g] Hard wheat flour, General Mills, Inc., Minneapolis, MN.

MATERIALS & METHODS

Formulation and processing

As presented in Table 1, formulations of pregelatinized pasta were manufactured according to a one-half replicate 2^5 factorial design (Cochran and Cox, 1957) with qualitative factors of wheat type [semolina (Sem), farina (Far), durum wheat flour (Dur), hard wheat flour (HWF)] and with quantitative factors of disodium phosphate (DP) (0, 1.0%); wheat gluten (WG) (0, 5.0%); and glyceryl monostearate (GMS) (0.75, 2.0%). Treatments were divided into two separate blocks because two production days were required to complete processing of all formulations. To supplement control formulations included in the basic fractional factorial design, two additional treatments (102 and 103) processed from semolina or farina without additives other than 0.75% GMS were incorporated into the first block.

Pregelatinized pasta was manufactured with a continuous HTST extrusion processing system on a pilot-plant scale by a patented process (Wenger and Huber, 1986). Operating conditions for the preconditioner and extruder were monitored with a microprocessor-controlled system. Batch processing operations were used for preliminary production stages.

Wheat (68.0 kg per run) and selected additives, if included in a formulation, were weighed, premixed in a surge-bin, and placed in temporary storage. After purification of the dry ingredients in a cyclone, the resultant mixture was transported to a vibrating feeder and filtered through a 40-mesh screen to remove large particulate matter before it was introduced into the atmospheric preconditioning cylinder at 3.18 kg/min. Steam was metered into the preconditioning chamber at about 0.80 kg/min and water at 0.45 kg/min. The temperature was 95.0 ± 2.0°C. Double-straight paddles with shaft speed 150 rpm were used to manipulate the moistened mixture during preconditioning.

The barrel of the food extruder (Model TX-80, Wenger Manufacturing, Sabetha, KS) (nominal diameter = 80.0 mm; ratio of barrel length to diameter = 25.5: 1; 75-kw DC drive motor) was designed with co-rotating, intermeshing twin screws modified to include 8 segmented zones. The first segment provided an inlet for mixture of raw materials at 3.86 kg/min from the preconditioning cylinder. The sixth segment was evacuated at 30 kPa to release steam. The second, seventh, and eighth segments contained cooling water at 80.0 ± 5.0°C. Thermal oil in jackets surrounding the third through fifth segments was maintained at 115.0 ± 5.0, 100.0 ± 5.0, and 90.0 ± 5.0°C, respectively. Water was added to the system between the first and second segments at 0.69 kg/min while steam was injected into the third and fourth segments at 1.6 kg/min. Pressure monitored at the seventh segment was 2.6×10^3 and at the eighth, 5.5×10^3 kPa. The extruder screw was operated at 160 rpm.

Pasta was extruded through a single die into the shape of elbow macaroni (0.4-mm wall thickness) and was cut with a 200-rpm rotating single-bladed knife into 2-cm long tubes. Cut samples were transported by a series of moving belts and pneumatic conveyors to a continuous double-pass dryer/cooler (Series III, Wenger Manufacturing, Sabetha, KS). Pasta samples were retained in the dryer about 40 min at 60°C and were dehydrated to 12% moisture or less. The standard vacuum-oven method for cereal products (AACC, 1983) was used to determine moisture of dried pasta samples. After drying, samples were sealed in plastic bags and stored in lightproof containers at room temperature until testing.

Compression-extrusion testing

Samples of dried pasta (50 g; moisture-free basis) were added to deionized water (500 mL) heated to boiling (99.0°C) on the highest setting of a Thermolyne hot plate Type 2200 (Model HP-A2235M, Sybron Corp., Dubuque, IA). Samples were stirred 5 times after 1.0, 2.0, 3.0, and 4.0 min rehydration. After draining water from pasta through a sieve at 5.0 min rehydration, pasta was allowed to rest in the sieve an additional 30 sec to facilitate water removal. An Instron Universal Testing Machine Model 1122 (Instron Corp., Canton, MA) interfaced to a Microcon II microprocessor (Instron Corp., Canton, MA) was used to obtain maximum force and maximum energy values on triplicate samples from each treatment. A 75-g sample of rehydrated pasta was placed in an Ottawa Texture Measuring System (OTMS) extrusion cell with a perforated plate (initial contact area = 50 cm^2) and compressed to 15% of its initial height in a single cycle in a compression-extrusion test (Voisey and Larmond, 1973). A crosshead speed of 100 mm/min was used. A 500-kg load cell was used to test samples at full scale load setting of 50 kg. The compression cycle on a pasta sample was initiated exactly 1.0 min after the 5.0 min rehydration step.

Spectrophotometric determination of amylose content

Procedures for measurement of amylose were adapted to suit pregelatinized pasta based on a quantitative method originated by McCready and Hassid (1943). Duplicate samples of dry pregelatinized pasta (5 g; moisture-free basis) were rehydrated in deionized water (50 mL) by the same procedure followed for preparation of samples for Instron measurements. Drained pasta was transferred to a centrifuge tube to which deionized water was added to bring total volume to 50.0 mL. Centrifuge tubes containing drained pasta were agitated 20 min on a Burrell Wrist Action Shaker Model 75 (Burrell Corp., Pittsburgh, PA). Water used in rehydration of pregelatinized pasta was transferred quantitatively to another set of centrifuge tubes. Sample residue remaining on the sieve after draining was rinsed from the screen into the tubes with 10.0 mL deionized water. Centrifuge tubes were filled with additional deionized water to bring total volume to 50.0 mL.

After both sets of tubes were spun at 2800 rpm (1650 × g) 15 min in an IEC CENTRA-7R Refrigerated Centrifuge (International Equipment Co., Needham Heights, MA) operated at room temperature (25°C), the supernatant in each tube was decanted. Aliquots of supernatant from rehydration water (0.5 mL) and water that had contained drained pasta (1.0 mL) were transferred in duplicate to spectrophotometer tubes and diluted with 14.5 and 14.0 mL of deionized water, respectively. After 1.0 mL iodine solution (0.002 N iodine in 2.0% potassium iodide; Sigma Diagnostics, St. Louis, MO) was added to each tube, contents were diluted with 9.0 mL deionized water to yield a final volume of 25.0 mL. Samples reacted with iodine reagent at least 10 min. Then absorbance was measured with a Spectronic 20 spectrophotometer (Bausch and Lomb, Inc., Rochester, NY) at 600 nm.

Titrimetric determination of total organic matter

Procedures for determination of total organic matter (TOM) in water used to rehydrate the pasta and in surface material removable from fully hydrated pasta after immersion in water were modified from methods of D'Egidio et al. (1982). Pregelatinized pasta samples were prepared for analysis in an identical manner to procedures followed for spectrophotometric determination of amylose. Drained pasta was transferred to 50 mL deionized water at room temperature (25°C) and stirred 5 times after 3.0, 6.0, and 9.0 min immersion. After 12.0 min, immersed pasta was separated from water by draining through a sieve. Sample residue adhering to the sieve was transferred quantitatively into immersion water by rinsing with 10.0 mL deionized water. Duplicate aliquots (5.0 mL) were transferred immediately from rehydration and immersion solutions to 600-mL breakers and evaporated to

dryness at 80°C in an oven (Model 524, Precision Scientific Co., Chicago, IL) evacuated with a vacuum pump (Model 1405, Welch Co., Skokie, IL) at 25 mm Hg.

After samples were cooled to room temperature (25°C), 10.0 mL 1.0 N potassium dichromate (Fisher Scientific Co., Fairlawn, NJ) and 20.0 mL concentrated sulfuric acid were gently mixed with the sample residue for 1.0 min. After a mimimum holding time of 30 min, the mixture was diluted with 200 mL deionized water. Diluted samples, to which 0.5 mL indicator solution (0.5% diphenylamine) (Sigma Chemical Co., St. Louis, MO) in concentrated sulfuric acid had been added, were titrated with 0.5 N ferrous ammonium sulfate (Sigma Chemical Co., St. Louis, MO) until the color changed from deep violet to emerald green. Percentage of total organic matter in the residue was calculated by the following:

$$TOM = \frac{C(B - S)}{B} \quad (1)$$

where B = volume of 0.5 N ferrous ammonium sulfate in mL required to titrate a blank sample, S = volume of 0.5 N ferrous ammonium sulfate in mL required to titrate the experimental sample, and C = constant factor of 6.941.

Gravimetric determination of solid residue

Material released during rehydration of pregelatinized pasta and surface material released from fully rehydrated pasta were quantified by gravimetric determination of solid residue according to AACC (1983) procedures. Duplicate samples of pregelatinized pasta were weighed to the nearest 0.0001g and prepared according to standard procedures developed for titrimetric determination of total organic matter. Rehydration and immersion solutions which had been combined previously with rinsings from respective sample residues adhering to sieves, were transferred quantitatively to dried, cooled, and preweighed 100-mL beakers and heated 24 hr at 98°C and 25 mm Hg in a vacuum oven. After dried samples were removed to a desiccator for cooling, samples were reweighed, and percentage of solid residue (%R) was computed for each sample by the formula shown below:

$$\%R = \frac{W_r}{W_s} \times 100 \quad (2)$$

where W_r = weight of solid residue and W_s = sample weight on a moisture-free basis.

Measurement of water absorption index and rehydrated volume

Duplicate samples of pregelatinized pasta (10 g) were weighed to the nearest 0.01g, rehydrated in 100 mL deionized water as described above for Instron analysis, and reweighed to determine hydrated weight. Water absorption index (WAI) relates to percentage increase in weight after rehydration (Grzybowski and Donnelly, 1979) and was computed by the following:

$$WAI = \frac{W_2 - W_1}{W_1} \times 100 \quad (3)$$

where W_1 = dry weight and W_2 = hydrated weight.

Volumes of rehydrated samples were measured according to a liquid displacement method (AACC, 1983). A graduated cylinder with a capacity of 50 mL was filled with an exact volume of naptha (Sunnyside Corp., Wheeling, IL) (25.0 mL). Rehydrated pasta was added to the cylinder, and the volume of liquid displaced was computed and reported as rehydrated volume of the pasta sample.

Statistical analysis

Data generated from the factorial design of 2^n treatments with fractional replication were analyzed by the half-normal plotting technique Daniel (1959). An empirical cumulative distribution of orthogonal contrasts was plotted on a half-normal probability scale against observed values for orthogonal contrasts. According to Milliken and Johnson (1989), the half-normal plotting method was suitable for estimation of experimental error and identification of effects that most likely are significant so that factors of potential importance could be examined during subsequent experimentation.

Two techniques were used to generate single degree of freedom sums of squares necessary for application of the half-normal plotting procedure. They included: (1) analysis of variance procedure to obtain sums of squares for main effects and estimable two-factor interactions to give a total of 15 orthogonal contrasts from the one-half replicate of the original 2^5fractional factorial design and (2) separate computation of sums of squares for 2 selected contrasts orthogonal to the original 15 contrasts to account for supplementary treatments incorporated into the basic fractional factorial design. Absolute values of square roots of each computed value were calculated and rank-ordered. Square roots of sums of squares for each observed effect were plotted against an empirical cumulative distribution function of ordered values. Any point that departed from a straight line drawn from the origin through a majority of the points would indicate a significant effect by the half-normal plotting technique because the observed value for the square root of the sum of squares of the contrast was larger than expected from the cumulative distribution function. Significance of an orthogonal contrast was determined by inconsistency with the half-normal plotting method; therefore, no probability values were associated with effects. The procedure outlined above was simplified by use of a computer program written in SAS® (Statistical Analysis System, SAS Institute, Cary, NC) by Milliken and Johnson (1989). Significant effects were eliminated from subsequent plots to permit estimation of standard deviation from the graphs by measurement of the horizontal distance which corresponded to a vertical distance of 1.0 on the straight line that passed through the origin and remaining points on the graph (Daniel, 1959).

When main effects of Wheat1 and Wheat2 or the interaction of Wheat1 and Wheat2, represented by Wheat1*Wheat2, were found to be significant by the half-normal plotting technique, wheat type (WT) was assumed to influence the specific characteristic under evaluation. Although Wheat1 formed a contrast of *Triticum aestivum* with *Triticum durum* and Wheat1*Wheat2 formed a contrast of fine particle size with coarse particle size, main effects and interactions of Wheat1 and Wheat2 were not interpretable by direct means; therefore, a set of orthogonal contrasts was constructed to allow for comparison of 4 types of wheat on a paired basis. Supplementary treatments (102 and 103) were excluded from these contrasts. Significance of these contrasts was determined by dividing the single degree of freedom sum of squares for a contrast by the estimated standard deviation from the half-normal plots. Contrasts were considered significant when the computed value exceeded 2.0. In subsequent discussions, WT will be used to refer to factors involving Wheat1, Wheat2, or Wheat1*Wheat2.

RESULTS & DISCUSSION

Physical properties derived from compression-extrusion testing

Single degree of freedom sums of squares and mean values for selected physical properties of pregelatinized pasta as measured with an OTMS system are presented in Tables 2 and 3, respectively. Mean values for selected additives (GMS, DP, WG) at low (0.75, 0, 0%) and high (2.0, 1.0, 5.0%) levels are represented by the following symbols: $\bar{x}_{0.75}$ (0.75% GMS), $\bar{x}_{2.0}$ (2.0% GMS), $\bar{x}_0$ (0% DP), $\bar{x}_{1.0}$ (1.0% DP), $\bar{x}_0$ (0% WG), and $\bar{x}_{5.0}$ (5.0% WG). Mean values for pregelatinized pasta formulated with durum flour, farina, semolina, and HWF are indicated by the symbols ($\bar{x}_{Dur}$), ($\bar{x}_{Far}$), ($\bar{x}_{Sem}$), and ($\bar{x}_{HWF}$), respectively. Of additives included in formulations in this study, GMS exerted the most substantial impact on physical measurements of pregelatinized pasta during compression-extrusion testing. Increasing level of GMS from 0.75 to 2.0% in formulations of pregelatinized pasta reduced mean values for maximum force ($\bar{x}_{0.75}$ = 590 N; $\bar{x}_{2.0}$ = 334 N) and maximum energy ($\bar{x}_{0.75}$ = 2.23 N-m; $\bar{x}_{2.0}$ = 1.33 N-m).

In addition to GMS, WT significantly altered maximum force values of pregelatinized pasta formulations. Effects of WT on Instron properties primarily were attributable to differences in mean values for semolina ($\bar{x}_{Sem}$) and HWF ($\bar{x}_{HWF}$); for maximum force, $\bar{x}_{Sem}$ = 408 N and $\bar{x}_{HWF}$ = 515 N. A close relationship between sensory scores for firmness and instrumental force values (r = 0.99) was reported by Jacobi (1984) for cooked spaghetti tested with an OTMS test cell. If that holds true for pregelatinized pasta, then data from our experiment suggest that incorporation of HWF into pregelatinized pasta formulations would result in a firmer product than use of semolina. Other reports concur with these findings (Kaci, 1982; Sheu et al., 1967; Tejada, 1982; Wyland and D'Ap-

Table 2—Single degree of freedom sums of squares[a] for physical properties of pregelatinized pasta as measured from a compression-extrusion test

Effect[b]	Maximum force ([N]$^{0.5}$)	Maximum energy ([N-m]$^{0.5}$)
Contrast1	167.4	6.654×10^{-3}
Contrast2	6.950×10^{4}*	0.5606
Block	89.98	3.089×10^{-3}
Wheat1	3.359×10^{4}*	0.4105
Wheat2	31.32	7.661×10^{-3}
Wheat1*Wheat2	826.6	3.089×10^{-3}
DP	6663	7.307×10^{-3}
Wheat1*DP	469.1	0.04397
Wheat2*DP	5904	0.04626
GMS	2.630×10^{5}*	3.167*
Wheat1*GMS	1186	0.03009
Wheat2*GMS	728.2	0.01475
DP*GMS	6491	0.08979
WG	1.624×10^{4}	0.2570
Wheat1*WG	1.487×10^{4}	0.09929
Wheat2*WG	9104	0.08086
DP*WG	2388	0.1376
Dur vs Far	1.194×10^{4}	0.1712
Dur vs Sem	589.9	0.01024
Dur vs HWF	1.578×10^{4}	0.1530
Sem vs Far	1.784×10^{4}	0.2652
Sem vs HWF	2.248×10^{4}*	0.2424
Far vs HWF	268.1	5.102×10^{-4}

[a] Sums of squares designated with an asterisk indicate significance of the main effect, interaction, or contrast as determined by the half-normal plotting technique (Daniel, 1959; Milliken and Johnson, 1989).

[b] Contrast1 = 102 vs 103; Contrast2 = average of 102 and 103 vs average of the remaining treatments processed on the first day of production; Wheat1 = (Far+HWF) vs (Dur+Sem); Wheat2 = (Sem+HWF) vs (Dur+Far); Wheat1*Wheat2 = (Dur+HWF) vs (Far+Sem); DP = disodium phosphate; GMS = glyceryl monostearate; WG = wheat gluten; Dur = durum flour; Far = farina; Sem = semolina; HWF = hard wheat flour.

Table 3—Mean values[a] for physical properties of pregelatinized pasta as measured from a compression-extrusion test

Treatment no.	Maximum force (N)	Maximum energy (N-m)
101	607	2.30
102	682	2.33
103	664	2.44
104	763	2.83
105	319	1.39
106	434	1.75
107	696	2.57
108	361	1.31
109	239	0.87
110	301	1.31
201	334	1.40
202	521	2.06
203	583	1.97
204	297	1.22
205	383	1.45
206	441	1.74
207	550	2.14
208	572	2.18
Standard deviation[b]	70.6	0.319

[a] Triplicate measurements on pregelatinized pasta samples rehydrated independently on different days.

[b] Estimated by the half-normal plotting technique (Daniel, 1959; Milliken and Johnson, 1989).

polonia, 1982); in our studies, *Triticum aestivum* also was shown to yield firmer product than *Triticum durum* when spaghetti products were processed by conventional methods and tested for firmness by instrumental methods.

In our experiment, demonstration of a lack of difference in firmness between pregelatinized pasta products processed with durum wheats of differing granulations supported findings of Seyam et al. (1974) for conventionally manufactured spaghetti. By contrast, Abecassis et al. (1987) found that a lower level of firmness was associated with conventional pasta prepared from durum wheat flour than from semolina. Differences in viscoelastic properties between durum wheats of different extraction rates were attributed to intrinsic variability in biochemical composition and solubility of wheat proteins rather than to differences in granulation of raw materials. To confirm observations from our investigation, effects of cultivar, agronomic characteristics, extraction rate, and biochemical composition of milled fractions on physicochemical properties of HTST processed pasta products would need to be studied.

Under conditions of our investigation, other factors of wheat gluten and disodium phosphate that were varied in the pasta formulations produced no distinguishable differences in physical properties of the pasta as measured in the compression-

Table 4—Single degree of freedom sums of squares[a] for physicochemical properties of rehydration and immersion solutions used in preparation of pregelatinized pasta

	Rehydration water			Immersion water		
Effect[b]	Total organic matter (%)	Amylose content[c]	Solid residue (%)	Total organic matter (%)	Amylose content[c]	Solid residue (%)
Contrast1	0.01862	0.006183	0.01232	0.0007851	0.0009570	0.003240
Contrast2	0.1003	0.01056	0.9434	0.04166	6.600×10^{-6}	0.6700
Block	0.07057	3.525×10^{-5}	0.1156	0.002583	0.0006728	0.1249
Wheat1	0.2896*	0.001292	0.4264	0.05151	2.197×10^{-5}	0.1150
Wheat2	0.04982	0.0004126	0.004323	0.0008251	2.822×10^{-5}	0.02653
Wheat1*Wheat2	0.01130	0.003488	0.1216	0.01303	0.0004384	0.002244
DP	0.01747	0.002407	0.6565	0.003449	0.0002157	0.5011
Wheat1*DP	0.01345	0.001160	0.009458	0.006033	4.307×10^{-5}	0.004048
Wheat2*GMS	0.001240	0.0005204	0.08880	0.01868	5.166×10^{-5}	0.01547
GMS	0.3312*	0.02354*	1.940*	0.1190*	0.0002954	0.9878
Wheat1*GMS	0.007760	0.0005790	0.0007426	0.004796	0.0002157	8.270×10^{-6}
Wheat2*GMS	0.02158	0.0009571	0.05476	0.002881	9.385×10^{-5}	0.05250
DP*GMS	0.1075	0.0008087	0.08237	0.02253	6.104×10^{-5}	0.005644
WG	0.003953	0.0005204	0.2211	0.005741	8.800×10^{-7}	0.2437
Wheat1*WG	0.1624	0.001118	0.1519	0.03696	1.650×10^{-5}	0.05079
Wheat2*WG	0.01211	0.0008087	0.3329	0.002271	2.822×10^{-5}	0.2375
DP*WG	0.0001382	0.002724	0.1229	0.01627	0.0001064	0.0006064
Dur vs Far	0.09323	0.004512	0.2913	0.006366	0.0001320	0.07469
Dur vs Sem	0.05429	0.003150	0.01549	0.003647	0.0001221	0.02210
Dur vs HWF	0.04959	0.001582	0.2583	0.03269	5.000×10^{-5}	0.1260
Sem vs Far	0.2898*	0.0001221	0.1724	0.01965	2.000×10^{-7}	0.01553
Sem vs HWF	0.2077*	0.0002674	0.1473	0.05817	0.0003283	0.04256
Far vs HWF	0.006831	0.0007508	0.0009901	0.01020	0.0003445	0.006670

[a] Sums of squares designated with an asterisk indicate significance of the main effect, interaction, or contrast as determined by the half-normal plotting technique (Daniel, 1959; Milliken and Johnson, 1989).

[b] Contrast1 = 102 vs 103; Contrast2 = average of 102 and 103 vs average of the remaining treatments processed on the first day of production; Wheat1 = (Far+HWF) vs (Dur+Sem); Wheat2 = (Sem+HWF) vs (Dur+Far); Wheat1*Wheat2 = (Dur+HWF) vs (Far+Sem); DP = disodium phosphate; GMS = glyceryl monostearate; WG = wheat gluten; Dur = durum flour; Far = farina; Sem = semolina; HWF = hard wheat flour.

[c] Absorbance measured at a wave length of 600 nm.

Table 5—Mean values[a] for physicochemical properties of rehydration and immersion solutions used in preparation of pregelatinized pasta

Treatment no.	Rehydration water			Immersion water		
	Total organic matter (%)	Amylose content[b]	Solid residue (%)	Total organic matter (%)	Amylose content[b]	Solid residue (%)
101	0.69	0.079	1.12	0.34	0.038	0.66
102	0.66	0.206	1.11	0.20	0.075	0.52
103	0.46	0.095	0.96	0.16	0.031	0.44
104	0.46	0.131	0.88	0.09	0.029	0.56
105	0.92	0.018	2.24	0.46	0.064	1.50
106	1.15	0.090	2.21	0.37	0.056	1.21
107	0.32	0.154	1.13	0.13	0.064	0.62
108	1.06	0.025	1.91	0.32	0.044	1.03
109	1.06	0.032	2.59	0.55	0.059	1.62
110	0.84	0.026	2.33	0.44	0.056	1.86
201	0.86	0.022	2.26	0.35	0.041	1.36
202	0.58	0.022	1.73	0.50	0.030	1.08
203	0.61	0.174	1.04	0.19	0.028	0.57
204	1.17	0.034	1.86	0.59	0.051	1.02
205	0.48	0.031	1.96	0.19	0.041	1.22
206	0.72	0.048	1.38	0.39	0.035	0.72
207	0.56	0.081	1.50	0.33	0.034	0.91
208	0.45	0.119	1.33	0.25	0.045	0.76
Standard deviation[c]	0.217	0.0490	0.460	0.135	0.0141	0.420

[a] Total organic matter and solid residue based on duplicates of pregelatinized pasta samples rehydrated independently on different days. Amylose content based on 4 observations (2 measurements × 2 different days).
[b] Absorbance measured at a wave length of 600 nm.
[c] Estimated by the half-normal plotting technique (Daniel, 1959; Milliken and Johnson, 1989).

Table 6—Single degree of freedom sums of squares[a] for rehydrated volume and water absorption index of pregelatinized pasta

Effect[b]	Rehydrated volume ($[mL]^{0.5}$)	Water absorption index (%)
Contrast1	0.2812	22.23
Contrast2	2.377	478.6*
Block	3.285	52.93
Wheat1	1.723	180.0
Wheat2	0.03516	7.503
Wheat1*Wheat2	2.066	51.67
DP	0.1914	11.98
Wheat1*DP	0.3164	0.6515
Wheat2*DP	0.6602	5.579
GMS	0.003906	1936*
Wheat1*GMS	0.1914	6.638
Wheat2*GMS	1.410	16.21
DP*GMS	0.3164	19.19
WG	1.129	87.61
Wheat1*WG	0.03520	29.91
Wheat2*WG	1.723	104.7
DP*WG	4.785	1.534
Dur vs Far	0.007812	19.41
Dur vs Sem	1.320	9.897
Dur vs HWF	0.6328	130.5
Sem vs Far	1.125	57.02
Sem vs HWF	3.781	212.3*
Far vs HWF	0.7812	49.27

[a] Sums of squares designated with an asterisk indicate significance of the main effect, interaction, or contrast as determined by the half-normal plotting technique (Daniel, 1959; Milliken and Johnson, 1989).
[b] Contrast1 = 102 vs. 103; Contrast2 = average of 102 and 103 vs average of the remaining treatments processed on the first day of production; Wheat1 = (Far+HWF) vs (Dur+Sem); Wheat2 = (Sem+HWF) vs (Dur+Far); Wheat1*Wheat2 = (Dur+HWF) vs (Far+Sem); DP = disodium phosphate; GMS = glyceryl monostearate; WG = wheat gluten; Dur = durum flour; Far = farina; Sem = semolina; HWF = hard wheat flour.

Table 7—Mean values[a] for rehydrated volume and water absorption index of pregelatinized pasta

Treatment no.	Rehydrated volume (mL)	Water absorption index (%)
101	19.75	96.9
102	17.75	98.2
103	18.50	91.5
104	18.25	93.7
105	20.75	126.5
106	19.75	110.0
107	18.50	94.5
108	18.25	116.3
109	19.25	130.8
110	20.25	128.2
201	16.50	118.9
202	19.75	103.5
203	18.00	97.7
204	20.00	124.9
205	18.00	110.7
206	18.25	114.0
207	18.50	97.1
208	18.50	101.0
Standard deviation[b]	1.200	6.90

[a] Each mean value is based on duplicate measurements taken on pregelatinized pasta on different days.
[b] Determined by the half-normal plotting technique (Daniel, 1959; Milliken and Johnson, 1989).

extrusion test. Conversely, addition of wheat gluten to conventionally processed spaghetti products increased extensibility of pasta dough during extrusion (Irvine, 1978) and improved firmness and elasticity after the product was cooked (Breen et al., 1977; Kim, 1987; Matsuo et al., 1972). Perhaps elevated temperatures and high shearing effects encountered with HTST extrusion processing could have denatured the gluten and limited its functionality in the pregelatinized pasta.

Physicochemical properties of rehydration and immersion solutions for pregelatinized pasta

In Tables 4 and 5, single degree of freedom sums of squares and mean values for selected physicochemical properties of solutions used to rehydrate or immerse pregelatinized pasta are presented. Few changes in those physicochemical properties occurred when pregelatinized pasta was formulated with different types of wheat or with varying levels of additives.

WT significantly influenced total organic matter (TOM) content of water used for rehydration. High TOM values have been associated with increased surface disintegration of conventionally prepared spaghetti products that can result in sensory perception of stickiness (D'Egidio et al., 1982). Pregelatinized pasta formulated with semolina showed an increase in TOM value of the rehydration water ($\bar{x}_{Sem} = 0.96\%$) relative to treatments containing farina ($\bar{x}_{Far} = 0.58\%$) or HWF ($\bar{x}_{HWF} = 0.64\%$). Kim (1987) and Kim et al. (1986) demonstrated that an increase in cooking loss accompanied substitution of farina for semolina in spaghetti processed by traditional cold-extrusion methods. Other workers reported that a reduction in particle size of durum wheat increased cooking losses for conventionally manufactured pasta products (Seyam et al., 1974) or reduced surfaced disintegration (Abecassis et al., 1987). Neither of these findings was substantiated for pregelatinized pasta products in our experiment because TOM values and

percent solid residue were not significantly different when pasta prepared with semolina was compared with that prepared with durum wheat flour.

Raising levels of GMS from 0.75 to 2.0% increased TOM values ($\bar{x}_{0.75}$ = 0.60%; $\bar{x}_{2.0}$ = 0.89%) and percent solid residue ($\bar{x}_{0.75}$ = 1.4%; $\bar{x}_{2.0}$ = 2.1%) of rehydration solutions and also increased TOM value ($\bar{x}_{0.75}$ = 0.28%; $\bar{x}_{2.0}$ = 0.41%) of immersion solutions. In addition, GMS reduced absorbance measured at 600 nm ($\bar{x}_{0.75}$ = 0.0354; $\bar{x}_{2.0}$ = 0.0295) which provided an index of amylose released from the surface during rehydration.

Water absorption index and rehydrated volume

Single degree of freedom sums of squares and mean values for water absorption index and rehydrated volume are listed in Tables 6 and 7 for treatments of pregelatinized pasta that we examined. GMS and WT significantly influenced water absorption index (WAI) of pregelatinized pasta. In our study, a high level of GMS increased WAI ($\bar{x}_{0.75}$ = 99.3%; $\bar{x}_{2.0}$ = 121.3%). For pregelatinized pasta products formulated with HWF, water absorption indexes ($\bar{x}_{HWF}$ = 104.4%) were lower than corresponding WAI's of semolina samples ($\bar{x}_{Sem}$ = 114.8%).

Neither DP nor WG affected physicochemical properties of rehydrated pasta or of water used to rehydrate or immerse pregelatinized pasta. Evans et al. (1975) found that sodium polyphosphates produced deleterious effects on rehydrated volume, WAI, and percent solid residue of spaghetti processed by conventional methods. No significant effects on WAI or percent solid residue were observed when WG was incorporated into pregelatinized pasta at a level of 5.0% in our experiment. However, Breen et al. (1977) and Kim (1987) noted a reduction in cooking loss but a consequent decline in cooked weight when WG was added to spaghetti processed by conventional extrusion. Differences in levels of WG added or variations in processing conditions could account for differences in results between pregelatinized pasta and conventional spaghetti.

CONCLUSIONS

OF INGREDIENTS incorporated into formulations of pregelatinized pasta in our investigation, glyceryl monostearate (GMS) had the most substantial impact on physicochemical properties studied to characterize quality of pregelatinized pasta. From empirical observations of extrusion for manufacture of pregelatinized pasta, GMS also was identified as a critical processing aid that imparted essential flow properties for proper extrusion of the dough under HTST conditions.

Although changes associated with wheat type were minimized for most quality factors, fewer processing difficulties were encountered when durum or hard wheat flours were used. Irregular flow patterns in extruders often accompany use of coarsely ground raw materials such as semolina or farina.

There was no apparent advantage in incorporation of disodium phosphate into formulations since anticipated effects of this ingredient on rehydration rate and textural properties were not demonstrated. Similarly, influence of wheat gluten on physicochemical properties was minimal.

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Influence of Milling Method and Peanut Extract on *in vitro* Iron Availability from Maize and Sorghum Flour Gruels

CARL M.F. MBOFUNG and ROBERT NDJOUENKEU

ABSTRACT

Maize and sorghum flours were milled by four different methods (a traditional, semi-traditional, mechanical and industrial method). Iron availability was estimated by an *in vitro* technique. The total, soluble and ionizable (available) iron as well as the phytate and neutral detergent fiber content of the gruels varied with type of flour and also with amount of peanut used in the preparation. The ionizability of iron decreased with increases in phytate and neutral detergent fiber. Iron in the gruels from flours milled by the traditional method was more available than that in other gruels.

INTRODUCTION

IRON DEFICIENCY is a widespread disease especially among nutritionally vulnerable groups in many parts of the world (developed and developing) and in particular those in which dietary iron is supplied mostly from plant-based foods (WHO, 1975). The wide range of physiological and functional abnormalities which include reduced work output (Dallman, 1982), enzymatic (Youdim et al., 1975) and behavioural changes as well as some alterations in brain function (Cantwell, 1974) attributable to iron deficiency cannot be emphasised enough.

In many cases the occurrence of iron deficiency may be due to a frank lack of iron in the diets customarily consumed. In other cases, the deficiency may be the result of low bioavailability of iron from the diets consumed due to presence of such antinutritional factors as phytate, fiber and tannins which bind iron and thus impair its absorption from the intestine. Phytate and fiber are high in cereals and cereal-based foods and as a result the absorption of minerals from these foods have generally been reported to be relatively low (Hallberg and Solvell, 1967).

The availability of iron from foods is known to vary. Review of research in this area (Lee, 1982) suggests there are many gaps in knowledge of the influence of food processing and preparation on iron availability. In particular, effects of certain traditional African methods of food processing and preparation on iron availability have not been investigated.

In Cameroon, and in particular the Northern ecological zones and Provinces, cereals form the staple food and are consumed in several forms especially as gruels for weaning infants and for adults at breakfast. Processing of these cereals into flours as well as the method of their subsequent preparation into gruels could influence the nutrient and antinutrient content of the final product. Our work was carried out to determine the effect of these treatments (processing and preparation) on the bioavailability of iron from the products using *in vitro* techniques. Another objective was to obtain information which could be useful to consumers in their choice of nutritionally better methods for processing and preparation of maize (or sorghum) gruel, a widely consumed food preparation.

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MATERIALS & METHOD

FOUR DIFFERENT processing techniques of cereal milling commonly practiced within the Adamawa Province, Cameroon were studied. They were: industrial flour milling (IMF); traditional processing (TME); semi-traditional processing (STM), and direct mechanical milling (DME). An abridged schematic flow chart of the different methods is shown in Fig. 1. These methods differ in several aspects. In the DME process, impurities in the graines were removed manually and the grains were milled directly into flour in a mechanical grinder (HINRICHS TECHNICS— Hamburg W. Germany). In the TME method the grains were moistened with water and dehulled using a mortar and pestle. The grains were then washed and soaked in water an average of 12 hr before being milled into flour by pounding in a mortar and pestle. The STM method was similar to the TME; the only difference being that the mechanical grinder was used in place of the mortar and pestle for dehulling as well as for milling the grains into flour. In the industrial process (IMF) grains were cleaned, dehulled and degermed before milling into semolina and flour. All the steps were operated automatically along the milling plant (OCRIM S.P.A. — Cremona, Italy).

There are several ways of preparing gruels from cereal flours but only two of the most common methods were selected for study. These were the plain gruel method and the gruel-peanut extract method. In the plain gruel method about 100g of flour (maize or sorghum) was sieved through a 250mm sieve and a known quantity of fine flour was mixed into a slurry paste using water. The slurry paste was then introduced with stirring into a specific quantity of boiling water over

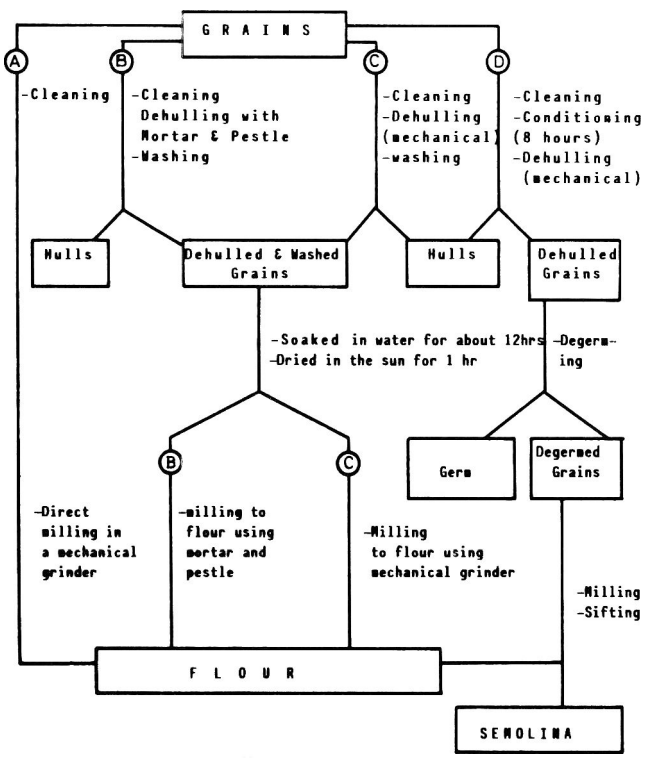

Fig. 1—Schematic flow chart of four different methods of milling maize and sorghum into flour: A = Direct milling method; B = Traditional method; C = Semi-traditional method; D = Industrial method

Table 1—Total, soluble, ionizable iron phytate and neutral detergent fibre (NDF) concentrations in plain maize and sorghum flour gruels as a function of method of milling

Gruel[a]	Total Iron mg/100g	% Soluble Iron	% Ionizable Iron	Phytate mg/100g	NDF g/100g
DMFG	1.76 ± 0.21[b]	22.0	1.40	500 ± 35[b]	32.0 ± 2.9[b]
TMFG	1.60 ± 0.13	32.0	19.00	230 ± 16	15.8 ± 1.2
STMG	1.20 ± 0.08	28.0	7.00	370 ± 22	19.3 ± 1.3
IMFG	1.06 ± 0.09	18.0	2.50	400 ± 34	22.0 ± 2.1
TSFG	3.10 ± 0.20	15.0	3.80	630 ± 43	33.0 ± 2.8
STSG	2.90 ± 0.24	24.0	10.00	300 ± 20	32.0 ± 1.9

[a] DMFG = Gruel of maize flour milled by direct method; TMFG = gruel of maize flour milled by traditional method; STMG = gruel made from maize flour milled by semi-traditional method; IMFG = gruel of industrially milled maize flour; TSFG = gruel made from sorghum flour milled by traditional method; STSG = gruel made from sorghum milled by the semi-traditional method.
[b] Means ± SD; n = 4.

the fire and allowed to cook while stirring 5 to 10 minutes. The use of sugar to sweeten the gruel is a common practice but for our study, no sugar was added.

In the gruel-peanut extract method flour was sieved in the same manner as above, and a known quantity mixed into a slurry and equally cooked into a consistent gruel-mixture using water into which peanut paste had been extracted. To assess the influence of added peanut in this preparation method, gruels were prepared using graded amounts of peanut paste (0, 30, 50, 100% by weight of flour) extracted into water. The different quantities of peanut paste were arbitrally chosen such as to include the quantity (estimated from observation and interview of women in the village to be about 30% by weight of flour) commonly used in the village. The peanut paste was produced by grinding lightly heated and decoated peanuts into a fine smooth feel in a mechanical grinder (MOLINEX, France). All gruels were prepared in duplicate.

The *in vitro* method adopted for the study was that of Narasinga Rav and Prabhavathi (1978) for determination of available non heme iron in foods. The method is based on the release of ionizable iron from foods by subjecting it to pepsin-HCl digestion at a simulated stomach pH of 1.35 and subsequently adjusting pH to 7.5 to simulate intestinal pH and then further digesting it with a pancreatin-bile extract. The ionizable iron measured at the pH of this last step (pH 7.5) has been shown to highly correlate with available iron absorbed *in vitro* (Narasinga Rao and Prabhavathi, 1978).

All glassware used was properly washed with liquid detergent, rinsed several times with distilled water and soaked 6 hr in 1N HCl before finally rinsing with copious amounts of distilled water. Only distilled water containing no traces of iron was used in the study. All chemicals used were reagent grade supplied by Sigma Chemical Co. (St. Louis MO); these included pepsin (EC 3.4.23.1); pancreatin (porcine pancreas grade VI); 2, 2' dipyridyl — and bile extract (porcine). Pepsin-HCl mixture contained 5g pepsin suspended in 0.1M HCl and brought to a total volume of 100 ml. For the pancreatic/bile mixture, 4 g pancreatin together with 25g of bile extract were dissolved in 0.1M sodium bicarbonate made up to 1L with 0.1M $NaHCO_3$.

Gruel samples prepared as described above, were dried to constant weight at low temperature (60°C), pulverized in a stainless steel desktop grinder and passed through a 250 mm sieve. The powder was then used for determination of total, soluble and ionizable iron, as well as the phytate and NDF content.

Two grams of each gruel sample were mixed with 25 mL pepsin-HCl and the pH adjusted to 1.35 before incubating in a 100 mL conical flask at 37°C in a shaker water bath 90 min. Following incubation, the digested sample was centrifuged at 3000 rpm 45 min and the supernatant carefully pipetted into another flask mixed with an equal volume of pancreatin-bile mixture. pH was adjusted to 7.5 with NaOH and the mixture reincubated at 37° another 90 min with shaking followed by centrifugation at 3000 rpm 45 min. The supernatant was used for analysis of soluble and ionizable iron.

The soluble iron of the supernatant was determined by the method of Tennat and Greeman (1969) while ionizable iron (free form of iron in the supernatant) was determined using 2,2'-dipyridyl as described by AOAC (1965). Total iron in each gruel type was determined by analysing aqueous solutions of ashed samples of the gruels also following the AOAC (1965) method. The accuracy of analysis of each batch of samples for total iron was monitored by similarly analysing Standard Reference Materials (Rice Flour-SRM 1568, obtained from the US Bureau of Standards, Washington) along with the samples in the batch. Phytate was determined by direct spectrophotometry after the method of Mohammed et al. (1986) while the neutral detergent fiber (NDF) was assayed by the method of Van Soest (1963) as modified by Reinhold and Garcia (1979). Statistical evaluation of data was carried out with the aid of a computer program, Statworks, (Cricket Software, PA 19104).

RESULTS & DISCUSSION

GRUELS, prepared (with or without peanut extract) from maize flours milled by four different methods and two sorghum flours also milled differently, were found to contain varying levels of iron (Table 1). Gruel (DMFG), prepared from maize flour milled by the direct mechanical method, had the highest iron content (1.76 mg/100g) while that prepared from industrially milled flour (IMFG) had the lowest (1.05 mg/100g). The gruels of sorghum flours contained relatively higher iron concentrations than did those of the maize flours; 3.1g/100g for gruels prepared from sorghum milled by the traditional method (TSFG) and 2.9mg/100g for gruel of sorghum flour milled by the semi-traditional method (STSG). The ionizable and hence the *in vitro* bioavailable iron from the different gruels varied with type of flour. On a percentage basis, iron was least available (1.4%) from DMFG and was highest (19%) from gruels (TMFG) prepared from maize flour obtained by the traditional processing method. The gruel of maize flour from the semi-traditional method (STMG) contained and 7% that from the industrial process (IMFG) 2.5% of available iron. Thus in relative terms, processing maize by the traditional method made iron about 14 times more available than from flour processed directly by the mechanical method. This apparent effect of method of milling on availability of iron seems in agreement with some reports in literature (Bjorn-Rasmusen et al., 1973) which illustrate the positive effect of milling on iron availability. It is possible however, that the level of iron (and indirectly percent available iron) in the flours milled by direct milling and semi-traditional methods could have been affected by iron contamination derived from mechanical abrasion in the mechanical grinder. This was not investigated. From the results of phytic acid and NDF analysis, flours with high phytate and NDF apparently contained less ionizable iron than those with low phytate and NDF.

For determination of effects of varying levels of peanut extract on availability of iron from the gruels, only two flour types, semi-traditionally milled maize flour and semi-traditionally milled sorghum flour were used. Analysing these gruels prepared with 0, 30, 50 and 100% by wet-weight of peanut

Table 2—Influence of the addition of graded quantities of peanut-paste to maize and sorghum gruels on the total, soluble, ionizable (available) iron, phytate and neutral detergent fibre (NDF) content

	Gruel							
	TMFG[a]				STSFG[a]			
% Peanut paste	0	30	50	100	0	30	50	100
Total iron (mg/100g)	1.60 ± 0.13[b]	2.40 ± 0.23[b]	2.94 ± 0.20[b]	4.30 ± 0.46[b]	2.51 ± 0.31[b]	3.30 ± 0.35[b]	3.68 ± 0.28[b]	4.68 ± 0.50[b]
% Soluble iron	32	36	30	28	24	26	25	24
% Ionizable iron	19	13	10	7.4	10	8.4	7.2	4.9
Phytate (mg/100g)	230 ± 18	300 ± 24	330 ± 20	1200 ± 85	330 ± 23	410 ± 28	780 ± 34	1400 ± 106
NDF (g/100g)	15.8 ± 2.1	22.8 ± 3.1	28.5 ± 2.6	35.8 ± 3.6	32.6 ± 1.9	39.0 ± 4.3	45.8 ± 4.1	56.8 ± 4.8

[a] TMFG = Gruel made from traditionally milled maize flour; STFG = gruel made from semi-traditionally milled sorghum.
[b] Means ± S.D.; n = 4.

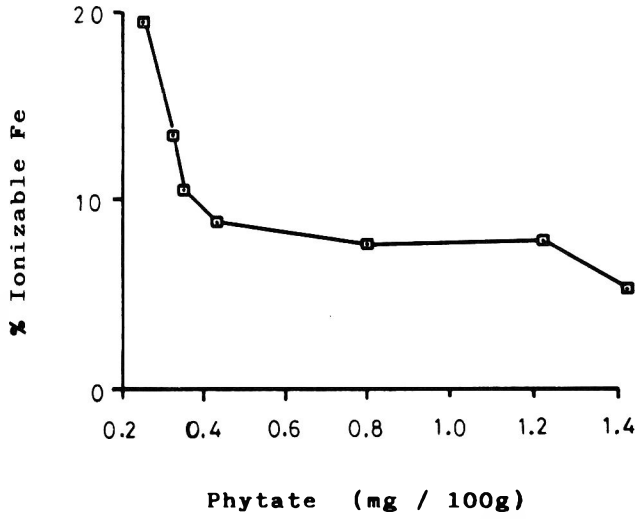

Fig. 2—Changes in percent ionizable iron with increase in phytate content of gruels prepared with peanut extract.

extracted into solution, showed a wide variation in level of total, soluble and ionizable iron and phytate of the gruels. Use of 100% by wet-weight of peanut extract raised the total iron content of the gruel by about 169% (i.e from 1.60 to 4.30 mg/100g). Although increasing concentration of peanut extract gruels raised the total iron of the gruels as well as absolute amounts of soluble iron, percent soluble and ionizable iron decreased with increased iron content of the gruels. Peanuts are a good source of plant protein and an increase in concentration of peanut extract in the gruel invariably denoted an increase in protein content. Several reported studies (Martinez-Torres and Layrisse, 1981; Schricker et al., 1982; Kane and Miller, 1984) tend to suggest observed differences in nonheme iron availability in meals containing different proteins could be due, at least in part, to the protein component. However, since no particular attempt was made in our study to evaluate the role of peanut proteins on availability of iron, we were not certain to what extent the protein content of the gruels, as influenced by the addition of peanut extract, contributed to ionizability of iron.

Analysis of the influence of phytate of the gruels on availability of iron also showed an increase in phytate was accompanied by a decrease in available iron ($r = 0.73$; $P < 0.04$). Recent observations by Hazel and Johnson (1987) have clearly emphasised this effect. A phytic acid content of between 0.40 and 0.78g% in the peanut containing gruels seemed to be the critical zone of phytate concentration. Below that a decrease in concentration had a high positive effect on iron availability and above that level an increase in phytate seemed to have little or no negative effect on iron availability (Fig. 2). Multiple regression analysis showed the percent ionisable iron (% ion Fe) in the gruels prepared with peanut extract could be predicted from the total iron (TFE), phytate (Phy), Fiber (NDF) and % peanut paste (PP) of any of the gruels by the equation:

$$\% \text{ ion Fe} = 28.13 - 5.97\text{TFE} + 0.006\text{Phy} - 0.119\text{NDF} + 0.021\text{PP}$$

with a coefficient of determination (R^2) of 0.97.

From the results of this study the technique of milling cereals as well as that of preparation influenced the concentration and bioavailability of iron from the maize and sorghum gruels. The composition of the flours milled was thus a function of efficiency of the method. The pattern of distribution of nutrients within cereals has been shown to vary within the main morphological parts of a cereal grain; most of the starch and proteins in maize are contained within the endosperm while most of the lipid and minerals are lodged within the germ (Kent, 1983). The effect of milling on these parts, therefore, directly affected the nutrient profile of the resulting flours. In the industrial process for example, degerming the grains, in addition to dehulling, removed a considerable quantity of nutrients from the final product. In the traditional method of milling, it was also possible that besides losses occurring in the nutrient content of the product through leaching into water during the 12 hr soaking stage, there could have also been some contamination of final product due to soaking and washing of the grain.

The observation of an inverse relationship between phytate and ionised iron levels is in agreement with reports in the literature (Hussain and Patwardham, 1959; Cummings, 1978) on the anti-nutritional effects of phytate and iron. This observation also suggested the effect of milling on iron availability. In fact early studies reported (Björn-Rasmussen, 1974) that availability of iron from wheat bread made from flours of different extractions varied in proportion to the bran (and by implication phytate and fiber) content of the flours. The variation in phytate and fiber content of the flours strongly suggested the different methods of milling differed in their effectiveness of dehulling. The semi-traditional technique of flour milling tended to produce flours that made gruels with relatively higher ionizable iron. It is likely that Besides the fact that mechanical methods of dehulling took off more of the phytate containing fractions of the grains, the soaking of the grains for upwards of 12 hr likely also contributed to decrease in phytate through activation of phytase. Although we did not investigate this aspect, it has been reported by other workers that long soaked and germinating grains tended to have increased activity of phytase (Beal and Mehta, 1985) and hence reduction in phytate content.

In conclusion, results of our study suggested that method of milling maize or sorghum into flour could influence nutrient content of the flours as well as the availability of iron from its products. Flours with high phytate and Neutral detergent fiber seemed to contain less ionizable iron than those with lower phytate and fiber. The method of preparation of maize or sorghum foods was equally important; use of peanut extract in the preparation of maize and sorghum flour gruels enhanced total and soluble iron in the gruels. The effect on ionizable iron content of the gruel was however, not uniform. The percent ionizable iron was relatively higher in gruels prepared from flours milled by the traditional method than from those milled by other techniques. Since iron deficiency anaemia is a very common nutritional problem in Africa, the significance of this work cannot be emphasized enough. The information so far obtained would be useful for public health education. The use of traditionally milled flour together with peanut extract in preparation of gruels would be beneficial to the consumer from the view point of iron nutrition.

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Rheological Characterization of Crosslinked Waxy Maize Starch Solutions under Low Acid Aseptic Processing Conditions using Tube Viscometry Techniques

ROBERT V. DAIL and JAMES F. STEFFE

ABSTRACT

A rheological characterization of 1.82 and 2.72% (g dry starch/100g water) waxy maize starch solutions was performed at 121.1, 132.2, and 143.3°C using tube viscometry techniques. Dilatancy was observed in 22 out of 23 experiments and the flow behavior index increased with concentration and decreased with temperature. Rheological behavior was explained in terms of the rigidity and volume fraction of the swollen granules combined with small shear stresses in the fluid due to the high temperatures and low shear rates. Parameter correlation analysis showed a near correlation between rheological parameters with the flow behavior index being the dominant parameter. The effect of dilatancy on residence time and heat transfer in the aseptic processing of particulate and nonparticulate foods is discussed.

INTRODUCTION

A PRINCIPAL barrier to approval of aseptic thermal processes for low acid particulate foods is the lack of basic information on flow behavior and convective heat transfer characteristics of liquid particulate mixtures (Sastry et al., 1989). Most recent attempts at obtaining fluid-to-particle convection coefficients have been done by solving the inverse conduction problem, i.e., measuring the internal temperature of a particle with known thermo-physical properties and estimating the boundary condition (Sastry et al., 1989; Chandarana et al., 1989). This is the most economical method of obtaining convection coefficients, and enables one to obtain the average convection coefficient as a function of fluid velocity past the particle. Also, with this method, the rheological properties of the fluid surrounding the particle are not required. However, for convection coefficients to be useful for hold-tube design, the relative velocity between the fluid and the particle must be known, and these calculations require that the rheological properties of the carrier fluid be known. These properties also enable solution of other problems pertinent to aseptic processing: determination of velocity profiles in hold-tubes, particle residence time and residence time distribution in hold-tubes and heat exchangers, and the critical Reynolds number for turbulence considerations.

The rheological properties of water (a Newtonian fluid) are readily available, and Sastry et al. (1989) approximated relative velocities for a neutrally buoyant particle, free to move with the flow, in water under 100°C. Approximate relative velocities were used in a Nusselt relationship to obtain convection coefficients, which were compared with experimentally determined values. Chandarana et al. (1989) determined convection coefficients for particles fixed in the flow of non-Newtonian fluids using a starch solution at approximately 130°C; however, rheological properties of the starch solution were not reported at that temperature.

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A widely used thickener for the fluid phase of low acid foods (e.g. chili or beef stew) is crosslinked waxy maize starch which is used because it does not form gels upon cooling. Crosslinking gives waxy starches a shorter (less stringy) texture. Also, crosslinked starches are less soluble and have elevated gelatinization temperatures which enable them to better withstand the thermal abuse given low acid foods (Fennema, 1976). To date, the only rheological characterization of crosslinked waxy maize starch appears to be that of Colas (1986). The rheological tests were performed at 25°C, and all of the pastes were pseudoplastic (shear-thinning) fluids. Since rheological properties are temperature sensitive, the data obtained by Colas are not usable for low acid aseptic process design.

Rheological characterizations have been performed for other cereal starch in water systems including corn (Christianson and Bagley, 1983; Christianson et al., 1982; Evans and Haisman, 1979; Doublier, 1987), and wheat (Bagley and Christianson, 1982; Wong and Lelievre, 1982; Doublier, 1981, 1987). These investigations were performed at temperatures below 70°C, and, for the most part, pastes were pseudoplastic. A notable exception occurred in the works of Bagley and Christianson (1982), Christianson and Bagley (1983), and Christianson et al. (1982) where dilatancy (shear-thickening) was observed at low shear rates for starches that were not completely gelatinized or were cooked for short times. Dilatancy was attributed to small shearing stresses in the fluid (due to high temperature or low concentration combined with low shear rate) which were unable to deform the starch granules that were still somewhat rigid.

Rheological parameters of power law fluids are affected by changes in concentration and/or temperature. Evans and Haisman (1979) observed the flow behavior index to increase with increasing concentration, whereas Colas (1986) observed a decrease. Colas (1986) and Doublier (1987) observed the consistency coefficient to increase with concentration, and Doublier (1981) and Colas (1986) reported it decreased with increasing temperature.

None of the rheological characterizations reported earlier are in the low acid aseptic processing temperature range. Therefore, characterization of a crosslinked waxy maize starch solution under these conditions would be beneficial since engineering design of aseptic thermal processes is not possible without it. The overall objective of this research was to complete a rheological characterization of two crosslinked waxy maize starch solutions at low acid aseptic processing temperatures using tube viscometry techniques. Specific objectives included: (1) building and instrumenting a tube viscometer that would prevent boiling of a test fluid heated above the atmospheric boiling point, and (2) investigation of temperature, starch concentration, and their possible interaction effects on the rheological parameters.

MATERIALS & METHODS

Tube viscometer system

A tube viscometer system was constructed to perform rheological tests on starch solutions under low acid aseptic processing conditions

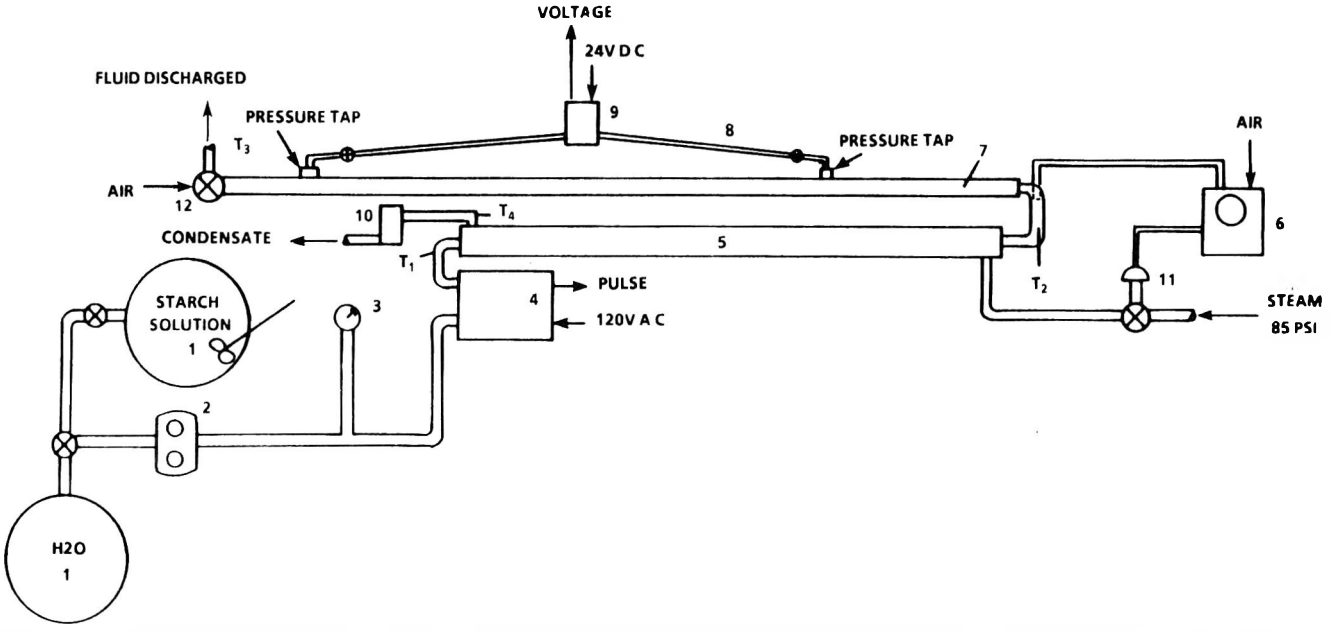

Fig. 1—Diagram of tube viscometer system: 1-Cherry Burrell UAS 50 vats; 2-Waukesha Model 10 positive displacement pump; 3 - Air filled shock tube with dial pressure guage; 4 - Micro Motion Model DL 100 mass flow meter; 5 - Concentric tube heat exchanger; 6 - Taylor Model D 121R Fullscope recorder/controller; 7 - Tube viscometer, 1.27 cm inside diameter; 8 - Stainless steel braid flexible hydraulic lines with bleeder petcocks; 9 - Taylor Model 405 T capacitive diaphram differential pressure transducer; 10 - Steam trap; 11 - Masoneilen "Little Scotty" air to open flow regulator; 12 - Cherry Burrell air to close flow control valve.

(Fig. 1). The distance between pressure taps was 4.59m and the diameter of the tube viscometer was 1.27 cm. The length from the entrance of the tube viscometer to the first pressure tap was 105 diameters (133.4 cm); consequently, end effects were assumed to have no effect on pressure readings. Complete details of the system were reported by Dail (1989).

Starch preparation, and a typical experimental run

The starch used in all experiments was National 465 supplied by National Starch and Chemical Corporation of Bridgewater, NJ (lot# DH4763). This starch is a crosslinked waxy maize recommended for low acid foods that are retorted or aseptically processed. Two concentrations were used: 1.815 and 2.723% on a dry basis (2 and 3% on a wet basis). Percentages are based on g starch/100g water, and are typical for the liquid portion of foods such as chili and beef stew.

To prepare the starch, the vat (Fig. 1) equipped with the motor and agitator paddle was filled with 156.2 kg of tap water at room temperature. The appropriate amount of starch (3.12 kg for 1.82%; 4.69 kg for 2.72%) was added to the vat in a manner which prevented clumping; a small portion of water was removed, and a starch slurry was created by slowly dispersing the starch into the water using a wisk. The slurry was immediately added back to the vat with the agitator paddle running which prevented settling of the starch while being pumped into the system. Tap water was used because of the large amount of starch solution required. The pH of the tap water was determined for each experiment. Starch was not gelatinized prior to introducing it into the system. A preliminary test to determine time required to achieve stable viscosity of starch gelatinized in the vat showed it increased in viscosity when maintained at 80°C. Since time required to execute each experiment was not constant, starch solutions were kept ungelatinized prior to testing, to insure the same starting material for each measurement within each experiment.

In a typical experiment the system was brought to operating conditions by pumping room temperature (25°C) water through it at 16 kg/min for 30 min. This allowed proper warm-up time for the mass flow meter, and the high flow rate insured evacuation of air bubbles from the system. After 30 min, the pump was set at the lowest setting, and the three-way valve was set to pump the starch solution into the system. The flow rate was increased to 16 kg/min for 4 min to insure displacement of all water. The system was then pressurized while simultaneously bringing the flow rate to the appropriate level for the first measurement by increasing the pump speed while closing the air-operated flow control valve (Fig.1).

To prevent boiling at the wall of the heat exchanger, the system was operated at a pressure between 552 and 690 kPa. Measurements were taken when the correct temperature and flow rate were achieved. The data acquisition system was programmed to record readings every 2 sec, and average them every minute. These averages were later retrieved. Temperatures of the starch solution entering and leaving the heat exchanger were recorded along with the mass flow rate, the pressure drop in the tube viscometer, and the temperature of the starch solution at the beginning and end of the tube viscometer. Flow rate was controlled with the air operated valve. A minimum of six measurements (six 1-min periods) were taken.

Experimental design

Two starch concentrations (1.82 and 2.72% db) at three temperature levels (121.1, 132.2, and 143.3°C) for a combination of six treatment levels, were examined. A two-factor (concentration and temperature), randomized, complete block design was chosen. Each block contains all six treatments so each block is a replication. Blocking was done over time to eliminate experimental or systematic effects occurring over time. Four blocks were executed for a total of twenty-four experiments.

Determination of rheological parameters

The experimental design was executed over a 14-day period. The pressure transducer and mass flow meter outputs were used to calculate pressure drops, volumetric flow rates, shear stress, shear rate, critical Reynolds number, and the generalized Reynolds number for the maximum flow rate of the experiment. These values are available elsewhere (Dail, 1989). Average pH of the tap water for all of the experiments was 7.73 ± 0.70 (95% confidence limits). An evaluation for the presence of slip was made using a larger diameter tube viscometer and the effect on shear rate was negligible.

Shear stress and shear rate values were calculated by the computer program "Tubev" developed at Michigan State University. The program calculates shear stress by substituting the pressure drop values in the following expression:

$$\sigma_w = \frac{\Delta PR}{2L} \tag{1}$$

Shear rate values were calculated using the Rabinowitsch-Mooney equation (Bird et al., 1987):

$$\dot{\gamma} = \frac{3Q}{\pi R^3} + \sigma_w \left[\frac{d\left(\frac{Q}{\pi R^3}\right)}{d\sigma_w} \right] \tag{2}$$

Table 1—Consistency coefficient, K (Pa s^n), and flow behavior index, n (dimensionless), from each experiment[a]

Experiment	Block 1	Block 2	Block 3	Block 4
121.1°C, 1.82% starch	$K=6.33*10^{-4}$ n=1.29	$K=3.49*10^{-4}$ n=1.41	$K=1.17*10^{-4}$ n=1.61	$K=6.37*10^{-4}$ n=1.27
132.2°C, 1.82% starch	$K=7.27*10^{-4}$ n=1.24	$K=5.28*10^{-4}$ n=1.30	$K=3.62*10^{-4}$ n=1.38	$K=2.43*10^{-4}$ n=1.46
143.3°C, 1.82% starch	$K=4.57*10^{-3}$ n=0.83	$K=1.69*10^{-3}$ n=1.07	$K=1.58*10^{-3}$ n=1.03	$K=2.37*10^{-4}$ n=1.44
121.1°C, 2.72% starch	$K=2.38*10^{-4}$ n=1.48	$K=4.34*10^{-5}$ n=1.86	$K=6.21*10^{-4}$ n=1.29	$K=1.39*10^{-4}$ n=1.59
132.2°C, 2.72% starch	$K=9.00*10^{-5}$ n=1.67	$K=1.83*10^{-5}$ n=2.08	$K=4.67*10^{-4}$ n=1.33	$K=2.77*10^{-4}$ n=1.46
143.3°C, 2.72% starch	failed	$K=1.17*10^{-3}$ n=1.11	$K=3.98*10^{-4}$ n=1.36	$K=2.57*10^{-4}$ n=1.47

[a] Each experimental block is a replication of six temperature/concentration combinations.

Table 2—Average consistency coefficient (K) and flow behavior index (n) for each temperature/concentration combination

Experiment	n_{avg}	$K_{avg}*10^4$ Pa s^n
121.1°C, 1.82% starch	1.40	4.34
132.2°C, 1.82% starch	1.35	4.65
143.3°C, 1.82% starch	1.09	20.2
121.1°C, 2.72% starch	1.56	2.60
132.2°C, 2.72% starch	1.63	2.13
143.3°C, 2.72% starch	1.31	6.08

Table 3—Flow behavior indices, consistency coefficients, and nonlinear coefficients of determination for each temperature/concentration combination (data have been pooled)

Experiment	n	$K*10^4$ Pa s^n	r^2
121.1°C, 1.82% starch	1.44	2.87	0.973
132.2°C, 1.82% starch	1.39	3.49	0.978
143.3°C, 1.82% starch	1.11	12.01	0.871
121.1°C, 2.72% starch	1.48	1.71	0.955
132.2°C, 2.72% starch	1.56	1.69	0.911
143.3°C, 2.72% starch	1.22	7.38	0.988

The computer program evaluates the derivative term (Eq. 2) by fitting $[Q/(\pi R^3)]$ versus σ_w with a polynomial equation and evaluating the derivative of the polynomial. The program also performs a regression analysis to estimate values of the consistency coefficient (K) and the flow behavior index (n) for the power law fluid model:

$$\sigma_w = K(\dot{\gamma})^n \quad (3)$$

This model was chosen because of its simplicity and excellent representation of experimental data (nonlinear coefficients of determination were between 0.934 and 0.998).

The shear rate range germane to aseptic processing was determined from flow rates and hold-tube sizes used in industry and found to be 10–75 sec^{-1} (Dail, 1989). This range is narrow for experimental purposes, so an attempt was made to cover a range of 10–150 sec^{-1}. The tube viscometer system was incapable of shear rates lower than 40 sec^{-1} due to inaccuracy of the mass flow meter at very small flow rates. The following shear rate ranges were covered for each temperature: 40 to 165 sec^{-1} at 121.1°C; 40 to 135 sec^{-1} at 132.2°C; 40 to 100 sec^{-1} at 143.3°C. The decrease in shear rate range as temperature increased was due to the inability of the heat exchanger to achieve the desired temperature at higher flow rates.

The theoretical basis for laminar-turbulent transition of Herschel-Bulkley and power law fluids in pipes is well established (Hanks and Ricks, 1974). The equations were programmed by Garcia and Steffe (1987), and their program was used to calculate the critical Reynolds number and the generalized Reynolds number for the highest flow rate. Flow behavior was laminar in all tests.

RESULTS

Analysis of variance

Estimated values of K and n (Table 1) were each treated as a single response for a particular experiment, and analysis of variance was carried out. The first analyses examined block and treatment effects for n and K, respectively. In both cases, block effects were not significant ($P=0.47$ for n and $P=0.28$ for K). Treatment effects were significant for n at $\alpha = 0.1$ (nearly 0.05) and for K at $\alpha = 0.05$. Therefore, changes in treatment levels (temperature and/or concentration levels) are the primary cause for changes in response (K and n values), and are not due to experimental or systematic effects. Since block effects were not significant, they were removed and the analyses were rerun showing treatment effects to be significant at $\alpha = 0.05$ for both parameters. Since block effects were insignificant the data from all four blocks for a particular treatment level (temperature/concentration combination) could be pooled, and/or K and n values averaged.

Examining temperature and concentration effects, along with possible interaction effects, on n showed the parameter to be significantly affected ($\alpha = 0.05$) by changes in both temperature and concentration. Interaction effects were not significant ($P=0.86$) so effects due to temperature or concentration change on this parameter can be examined independently. The flow behavior index was slightly more affected by changes in concentration ($P=0.03$) than by changes in temperature ($P=0.04$). A similar analysis on K yielded similar results; K was significantly affected by changes in temperature ($P=0.04$) with nearly significant temperature effects ($P=0.11$). Interaction effects were not significant ($P=0.29$).

Average values (treatment means) of n and K are presented in Table 2 for each treatment level. Generally, n decreased, and K increased, as temperature increased. However, for a given concentration, neither changed much until the temperature was increased to 143.3°C. Also, n increased, and K decreased, with increasing concentration.

Rheograms

The analysis of variance examining temperature, concentration and interaction effects was performed with the average values of K and n for each treatment level. Another way of viewing the results is to pool the data from the four blocks and perform nonlinear regression to obtain the rheological parameters. This allows a comparison between the parameters obtained from the pooled data and the treatment means. It also allows visual observation of the variation within a treatment level when plotted in a rheogram.

Nonlinear regression was performed, and rheograms were created, with commercially available statistical/plotting software called "Plotit" (Eisensmith, 1987). The pooled data parameters are presented in Table 3 along with the nonlinear coefficients of determination. A comparison with the treatment means in Table 2 shows that in most cases the values are nearly the same and follow the same trends. This is the expected result.

Rheograms show the combined effect of n and K, since both are required to define the curve. Figure 2 shows the temperature effect when concentration is held constant at 1.82%. The

Table 4—Sensitivity coefficients (m^3/sec) and their ratio for various values of pressure drop (Pa) when n = 1.5 and $K = 5.0*10^{-5}$ Pa s^n

ΔP Pa	$K(\partial Q/\partial K)*10^6$ m^3/sec	$\eta(\partial Q/\partial \eta)*10^6$ m^3/sec	Ratio*
50	−2.465	−9.801	3.976
100	−3.912	−18.21	4.655
150	−5.126	−25.95	5.062
200	−6.210	−33.23	5.351
250	−7.260	−40.17	5.575
300	−8.138	−46.84	5.756
400	−9.858	−59.57	6.043
500	−11.44	−71.69	6.267
600	−12.92	−83.30	6.447
700	−14.32	−94.55	6.603

* Column 3 divided by column 2.

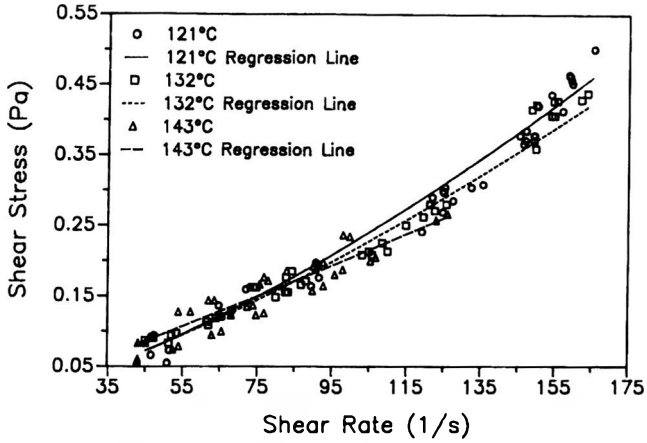

Fig. 2—Temperature effect with 1.82% starch concentration.

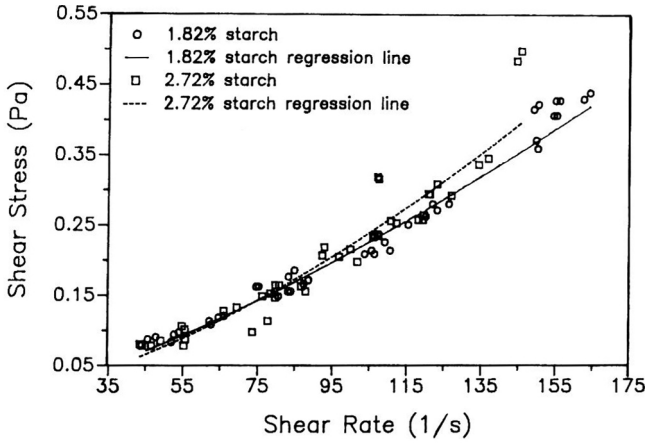

Fig. 3—Concentration effect at 132°C.

depressing effect of increased temperature on n is apparent. Figure 3 shows the concentration effect when temperature is held constant at 132.2°C, and the elevating effect on n with increasing concentration is apparent.

Parameter correlation analysis

Nonlinear regression analyses showed parameter correlation matrices yielded values near one for the off diagonal elements indicating that n and K might be correlated. If absolutely correlated, one of the parameters should be eliminated by expressing it as a function of the other creating a one parameter model. To investigate this possibility, the following sensitivity coefficients were determined and plotted (Beck and Arnold, 1977):

$$(K)\frac{\partial Q}{\partial K} \qquad (n)\frac{\partial Q}{\partial n}$$

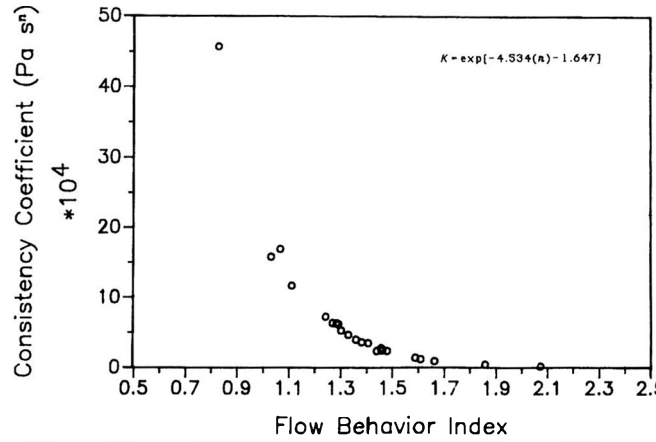

Fig. 4—Consistency coefficients versus flow behavior index.

An expression for the volumetric flow rate, Q, was obtained by creating an alternative version of the power law model:

$$\frac{\Delta PR}{2L} = K\left[\left(\frac{3n+1}{4n}\right)\left(\frac{4Q}{\pi R^3}\right)\right]^n \qquad (4)$$

Equation (4) was rearranged to solve the volumetric flow rate explicitly, and the derivatives were obtained from this expression. Sensitivity coefficients were calculated for values of pressure drop between 50 and 700 Pa for two values of n (1.0 and 1.5) and a constant value of K equal to $5.0*10^{-5}$ Pa s^n. These values are within the range in the experiments. Sensitivity coefficients, for the case when n = 1.5, are presented in Table 4. Similar results were found with n = 1.0.

If the sensitivity coefficients are linearly dependent, then the parameters are absolutely correlated (Beck and Arnold, 1977). In this study, they did not prove linearly dependent, but are nearly so, as indicated by the ratio of sensitivity coefficients (Table 4). Therefore, both parameters are required to describe flow behavior. Near correlation makes it impossible to unambiguously estimate the parameters and makes it difficult to interpret their behavior.

An objective of this research was to examine the influence of temperature and concentration on the rheological parameters. Unambiguous estimates of the parameters would seem to make analysis of variance meaningless. However, the fact that the parameters were significantly affected by changes in concentration and temperature suggests one of the parameters is dominating the fluid behavior. An examination of Table 4 reveals n to be the dominating parameter, because small perturbations in n cause larger changes in the volumetric flow rate than the same perturbations in K. Consequently, the behavior of K is mostly determined by the behavior of n and not by changes in concentration and temperature.

Further evidence of near correlation of the parameters can be seen in Fig. 4 where a plot of K versus n is presented. An equation that fits this relationship well is

$$K = \exp[-4.534(n) - 1.647] \qquad (5)$$

Using the first three values of n from Table 3 in Eq (5) yields values of $2.76*10^{-4}$, $3.55*10^{-4}$, and $12.50*10^{-4}$ Pa s^n for K, respectively. These values compare favorably to the estimated values in Table 3: $2.87*10^{-4}$, $3.49*10^{-4}$, and $12.01*^{-4}$ Pa s^n, respectively. However, not all of the changes in K can be predicted by n, so it is inappropriate to incorporate Eq (5) into the power law model to create a new, one parameter model. The inverse relationship between the parameters demonstrated in Fig.4 can also be seen in the parameter values (Table 2): as n increases, K decreases and vice-versa. The influence of n on K makes the analysis of variance with K

difficult to interpret, since values of K are essentially determined by n.

DISCUSSION

Near correlation of the parameters

Near correlation of the parameters serves as an explanation for the unexpected responses (increasing with increasing temperature and decreasing with increasing concentration) of K in this study. This might have been avoided had the sensitivity coefficients been investigated beforehand, but this requires prior knowledge of the parameter values. Using a broader volumetric flow rate range (and consequently, shear rate) would also help avoid the problem; however, this would have been difficult to achieve because of the laminar flow requirement of the Rabinowitsch-Mooney equation (Eq 3). Also, broadening the shear rate range far beyond that seen in aseptic processing may have made the results useless.

Near correlation of parameters has no affect on predicting hold-tube velocity profiles; the only loss is the physical significance typically ascribed to each parameter. Usually, when parameters are almost correlated, they must be used together, because estimates of them individually are unreliable. Estimates of n indicate shear-thickening fluids in this study. Observation of the data points (Fig.2 and 3) show shear thickening behavior. Consequently, n has retained most of the physical significance typically attributed to it.

Effect of dilatancy on hold-tube velocity profiles and its implications for aseptic processing

Shear-thickening behavior results in the velocity differential between the fastest and slowest moving fluid stream being greater than if the material was shear-thinning. This is true for both laminar and turbulent flow (Dodge and Metzner, 1959). Mathematically, for laminar flow of power law fluids in tubes:

$$U_{max} = \left[\frac{(3n + 1)}{(n + 1)}\right] U_{avg}$$

Therefore, the fluids in this study (or any dilatant fluid) have maximum local velocities that are greater than twice the average velocity when in laminar flow. Laminar flow will be found only in some pilot scale systems. Using flow rates and hold-tube sizes typically used in industry shows that most commercial systems would be in turbulent flow. For instance, a system filling 176 (227g) containers per minute with a 0.0635m diameter hold-tube shows a generalized Reynolds number of approximately 12,000 when the properties for 1.82% starch at 143°C (Table 3) are used. Many commercial systems have hold-tube lengths based on a Newtonian laminar flow assumption ($U_{max} = 2U_{avg}$), even if the fluid being processed is non-Newtonian. Consequently, the potential exists for significant improvement in product quality, since the velocity profile for turbulent flow is much flatter than that for laminar flow.

The microorganism of concern in the thermal processing of low acid foods is *Clostridium botulinum* which requires that hold-tubes be designed based on the worst case value of n for each temperature/concentration combination. Therefore, the largest n values (Table 1) for each concentration/temperature combination should be used. Note that these results may only apply for the conditions of these tests: producers of low acid foods should perform their own rheological characterization for different processing conditions, which may include a change in pH, addition of salts, sugars, or hydrocolloids, and markedly different heat exchanger residence times.

In processing low acid foods containing particulate matter, dilatant flow behavior has several implications. First, the fluid next to the particles will be more viscous than the bulk fluid due to the velocity gradient in the boundary layer of the particle. This will decrease heat transfer rates because the more viscous fluid will act as an insulator. On the positive side, viscous drag on the particle should be increased which will aid in keeping the particle suspended and moving with the fluid. Dilatant behavior will also result in greater over-processing of the fluid phase than if the starch solutions were shear-thinning, because the differential is greater between the fastest moving particle and the slowest fluid stream. Since heat is being transferred from the fluid to the particle, it is doubtful that producers of foods that contain large particles will realize improved product quality. Given the other problems in developing thermal processes for particulate-containing foods, producers should consider whether current aseptic processing technology is appropriate for foods containing large particles when the carrier fluid is dilatant.

Dilatancy and changes in the flow behavior index (n)

The fact that n was observed to increase with concentration agrees with the results of Evans and Haisman (1979) but disagrees with those of Colas (1986). The increase with concentration and decrease with temperature observed in this study can be explained with the same logic used by Bagley and Christianson (1982), Christianson et al. (1982), and Christianson and Bagley (1983): increasing the concentration causes the granules to become more closely packed and increases n if the granules are still somewhat rigid. They observed rigidity in starch granules for low cook temperatures where all granules had not gelatinized and/or cook times were short.

In the current study, the starch was cooked in the heat exchanger, and the cook time was the residence time in the heat exchanger. If the fluid is assumed Newtonian and an average density value is used, an estimate of the average residence time can be obtained. This showed minimum residence time of approximately 17 sec, and a maximum at approximately 144 sec. With such short cook times, possibly little amylopectin is solubilized, and the granules are still rigid. Colas (1986) showed that the close-packing concentration for unmodified waxy maize was 1.76% and was 2.60% (g dry starch/100g water) for the most lightly crosslinked waxy maize. Starch concentrations used in this study are within, or exceed, this range. Also, close-packing concentration decreases with increasing temperature (Bagley and Christianson, 1982). Thus, if the granules in this study were still rigid, it is reasonable to expect n to increase with concentration. This, combined with the fact that high temperatures and low shear rates caused the shear stresses in the fluid to be very small, provides the best explanation for the observation of dilatancy. The fact that n decreased with increasing temperature might be expected, because the granules would imbibe more water making them less rigid and more likely to deform under stress.

The observed dilatancy is not likely to be due to ongoing gelatinization and/or pasting; i.e., the fluid is not strongly time-dependent. That the gelatinization range for cross linked waxy maize starches is 72–82°C (Hoseney, 1986) is well documented. Therefore, incomplete gelatinization is not likely, even at the highest flow rate. Ongoing pasting may have affected the results. However, for this to occur, significant solubilization would have to occur between the tube viscometer pressure taps. Since this starch is all amylopectin, which solubilizes much more slowly than amylose, and the residence time between the pressure taps is approximately 144 sec at most, we believe the effect of ongoing pasting is minimal.

The amount of granule swelling depends only on temperature and changes in viscosity over time are due to solubilization of the starch granule (Christianson et al., 1982). Therefore, different gelatinization rates (due to varying flow rates) did not affect the results reported in this study, i.e., the fluids did not have different rheological properties at each flow rate. Contrary results have been reported by Härröd (1989) in a study involving potato starch. Since potato starch has a high amylose

fraction (and amylose starts solubilizing at the gelatinization temperature), his results may have been influenced by solubilized amylose.

The temperature dependence of n has several implications for aseptic processing. First, for processors of particulate foods where there is cooling of the liquid phase as the particles are being heated, the rheology of the suspending solution will change as the solution temperature changes. In turn, the velocity profile and residence time of the fastest moving particle will change. This effect becomes greater with increased percentage of particles. If the change in fluid temperature is known as a function of hold-tube length, it is possible to recalculate the velocity profile along the length of the hold-tube. An alternative, microbiologically conservative approach would be to design the hold-tube on the basis of constant rheological properties using the highest n value, i.e., that corresponding to temperature at the end of the hold-tube. This would result in some over-processing. The flow behavior of the starch used in this study (National 465) is more sensitive to temperature change between 132 and 143°C. Hence, over-processing based on assumed constant rheological properties in the hold-tube is minimal when hold-tube temperature is between 121 and 132°C.

A second implication of the temperature dependence of n is that the differential between the fastest and slowest fluid stream is minimized for higher temperatures, since n generally decreases with temperature (Table 2). This means that processors should obtain higher quality product from processing at higher temperatures.

The concentration dependence of n indicates a higher quality product will be obtained with lower starch concentrations, since this will minimize the differential between the fastest and slowest fluid streams. For the material in this study, the highest quality will be obtained for product formulated with the lower starch concentration, processed at higher temperature. This is particularly true for nonparticulate foods or foods with small rapidly heating particles. In foods that contain large, slow heating particles (e.g., meat pieces), the quality gained by minimizing the differential between the fastest and slowest fluid streams by processing at higher temperatures will be lost to the extra time at the high temperature required to sterilize the particles.

SUMMARY & CONCLUSIONS

A RHEOLOGICAL CHARACTERIZATION of two waxy maize starch solutions (1.82 and 2.72% db) was completed, and the solutions were found to be shear-thickening (dilatant). Dilatancy was explained in terms of the rigidity and volume fraction of the swollen starch granules combined with exceedingly small shear stresses in the fluid due to the high temperatures and low shear rates.

Analysis of variance showed the flow behavior index (n) to be significantly affected by changes in both temperature and concentration: increasing with concentration and decreasing with temperature.

The construction of sensitivity coefficients showed the two power law fluid parameters to be nearly correlated with n being the dominant parameter. This made the observed behavior (and the analysis of variance) of K difficult to interpret, because most of its behavior is determined by n. However, because the correlation is not absolute, the estimated values of the parameters are unique.

The effect of dilatancy on power law fluid velocity profiles is to increase the differential between the fastest and slowest fluid stream. For low acid foods that contain discrete particles, this can cause greater over-processing of the fluid phase since the differential between the fastest moving particle and the slowest fluid stream is greater than if the carrier fluid was shear-thinning. A dilatant fluid will also act as an insulator because of shear-thickening in the boundary layer of the particle. Consequently, food processors should consider whether aseptic processing is appropriate for foods containing large particles. For nonparticulate foods, the highest quality product will be obtained for foods with lower starch concentrations processed at higher temperatures.

NOMENCLATURE

K	= consistency coefficient (Pa s^n)
L	= length (m)
n	= flow behavior index (dimensionless)
P	= probability of F ratio being greater than F
$\triangle P$	= pressure drop (Pa)
Q	= volumetric flow rate (m^3/sec)
R	= tube radius (m)
r^2	= coefficient of determination
U_{avg}	= average fluid velocity (m/sec)
U_{max}	= maximum fluid velocity (m/sec)
α	= level of significance
$\dot{\gamma}$	= shear rate (s^{-1})
π	= circumference of circle divided by its diameter
σ	= shear stress (Pa)
σ_w	= wall shear stress (Pa)

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Evaluation of Emulsion Stability by Centrifugation with Conductivity Measurements

B. LATREILLE and P. PAQUIN

ABSTRACT

An industry problem when evaluating stability of emulsions is lack of a method reproducing, in a short time, aging of the emulsion. A method that combines centrifugation and conductivity measurements could be used for this purpose. Centrifugation accelerates destabilization of the product and thus simulates aging. Conductivity measurement of the aqueous phase, a function of the oil concentration, evaluates destabilization. Our method was compared with a creaming-measurement method based on fat content. The increase in conductivity was representative of the creaming rate, with correlation higher than 0.93. The combined method, applied to different oil-in-water (O/W) emulsions, enables following stability during aging and assures the need of industry for a fast, easy method to evaluate emulsion stability.

INTRODUCTION

IMPROVING and maintaining the physical stability of emulsions, such as UHT milk, cream liquor or table cream, is a major need of the food idustry. For milk-based infant formulae, with a shelf life of 12–18 months, Mc Dermott (1987) indicated lack of adequate physical stability during that time could be misinterpreted as microbial spoilage, and is aesthetically unacceptable.

The most common instability in oil-in water emulsions is separation of the dispersed phase due to the difference in density between the phases. This physical instability can arise through various mechanisms such as creaming, flocculation or coalescence (Mulder and Walstra, 1974).

Accelerated tests are employed to predict the stability of an emulsion for a long time under normal conditions (Sherman, 1971). Acton and Saffle (1970) incubated emulsions at 37°C for 24 hr and expressed stability in terms of moisture content in the bottom 5 mL sample as compared with the initial moisture content of the emulsion. Smith and Dairiki (1975) determined emulsion stability by holding them for 24 hr at 23°C and expressed stability based on total fat in the bottom half after storage, relative to total fat in the original sample. Similarly, Tornberg and Hermansson (1977) used the relationship between fat in the centrifuged lower phase and that in the original emulsion as a stability index. Yasumatsu et al. (1972) as well as Wang and Kinsella (1976) used the amount of fat released after centrifugation of heated emulsions as an index of stability. Petrowski (1974) studied emulsion stability by measuring free oil after centrifugation at 37,000 × *g* for 10 min and evaluating the dielectric constant of the product after microwave treatment. These methods are laborious and time consuming.

To better evaluate the stability of emulsions during storage, the industry needs an easy to use method which simulates acceleration of the aging process and reduces the time to evaluate stability of fresh products.

According to Becher (1965) if we assume that stability is directly proportional to gravitational force, long-term behavior of the emulsion could be assessed by centrifuging at moderate speeds. According to Stoke's law, the centrifugation acts on the emulsion by destabilizing the dispersed fat resulting in a rise of fat globules (Kessler, 1981). Conductivity is of interest as a means of monitoring the destabilization level of emulsions storable for long periods. The fat globules of milk reduce conductivity by occupying volume and by impeding mobility of ions (Prentice, 1962). Kato et al. (1985) proposed evaluating emulsifying properties (capacity and stability) of short shelf life (5 min) model emulsions by conductivity measurements.

Applying a simple and rapid method combining centrifugation and conductivity measurement, the objective of our work was to compare stability level of complex emulsions submitted to accelerated aging. To verify its accuracy, the method was compared to a method that evaluates emulsion stability by determining the concentration of fat at different levels in the product.

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MATERIALS & METHODS

Infant formula

We used a concentrated milk-based liquid infant formula, processed as a commercial product. The basic composition was: 7.2% fat, 14% carbohydrates, 3.1% proteins and 0.72% minerals and vitamins.

The ingredients used were: water, skim milk, lactose, vegetable oil (coconut and soybean oil), skim milk powder, emulsifiers (monoglycerides, diglycerides and lecithin), stabilizers (carrageenan) and various minerals and vitamins.

Water and ingredients were mixed in a tank. After light homogenization, the pH of the mix was adjusted to 7.2. The final homogenization was executed using a M-110 microfluidizer® (Microfluidizer Corp., 1985). The microfluid was used at pressures of 19.3, 48.3 and 77.2 megapascals (MPa) and at 70°C. Finally, the product was canned and commercially sterilized.

Conductivity measurements of the emulsion

The conductance of the infant formula (native emulsion) was measured using a conductivity electrode (YSI Co. Inc., model 3402, Yellow Spring, OH) modified by removing its protective chamber and placing in a graduated cylinder (3 cm diam) for measurements. The electrode, fixed to a vertically movable support permitting immersion to the same depth (3.2 cm from the bottom of the cylinder), was connected to a conductance meter (YSI Co. Inc., Model 35, Yellow Spring, OH). A diagram of the apparatus used for conductivity measurements is presented in Fig. 1. Note that the protective chamber was removed to facilitate stirring the product and cleaning the cell and the electrode. Furthermore, the electrode position and the graduated cylinder size are critical to avoid wall effects on conductance measurements. These precautions were established from previous experiments in our laboratory.

Conductance measurements of native emulsions were carried out at 21°C. They were obtained after 60 sec on 30 mL samples, under constant stirring with a magnetic stirring bar (1.25 cm long, 0.3 cm diam) to provide homogeneous product.

For stability measurements emulsions were treated to simulate various degrees of accelerated aging by centrifugation. Aliquots of 150 mL from a freshly prepared infant formula were centrifuged at 2,000 × *g*, 4,000 × *g*, or 12,000 × *g* for 60 min each, at room temperature. Centrifugations were carried out using a Sorval centrifuge (Du Pont Instruments, model RC-5B, rotor GSA, Newtor, CT), at room temperature. After centrifugation, fat layers were observed on top of the samples. The lower phase (50 mL) of the centrifuged emulsion was

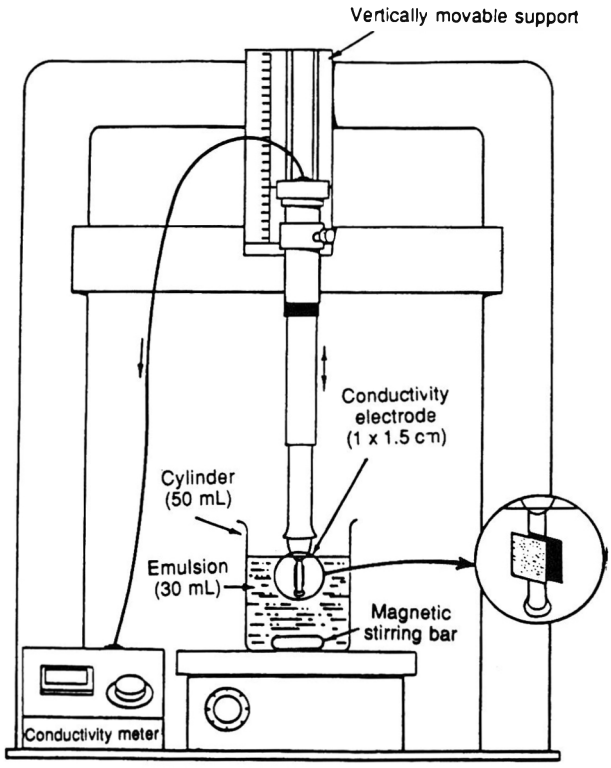

Fig. 1—Diagram of the apparatus measuring emulsion conductivity.

Table 1—Conductivity of the native emulsion (C_o) at different repetition

Trial no.[a]	Repetitions[b] 1	2	3	Mean ($m\Omega^{-1}cm^{-1}$)	Std. dev.
1	5.13	5.15	5.16	5.17	±0.03
	5.21	5.18	5.16		
2	5.03	5.08	5.10	5.07	±0.02
	5.08	5.05	5.07		
3	5.01	5.06	5.04	5.05	±0.04
	4.99	5.08	5.10		

[a] Refers to microfluidization pressures, 19.3, 48.3 and 77.2 MPa, respectively.
[b] Based on duplicate analyses of the product.
[c] Refers to the six determinations of each trial.

removed by gentle suction using the method of Maxcy and Sommer (1954) and conductance measured at 21°C as previously described.

The conductivity value ($m\Omega^{-1}cm^{-1}$) is obtained from the conductance ($m\Omega^{-1}$) and the electrode constant (approx. 0.1 cm^{-1}), experimentally determined with the procedure described by the manufacturer (YSI Co. Inc., 1983).

For each centrifuged sample, emulsion stability was estimated by calculating the difference (△ Conductivity) between the conductivity of the lower portion creamed by centrifugation (C_l) and the conductivity of the original emulsion (C_o):

$$\triangle \text{ Conductivity} = C_l - C_o$$

After each measurement, the electrode was cleaned using a 1:1 solution of methanol and chloroform (Anachemia, Montreal, Canada), and rinsed with distilled deionized water.

Stability index measurements of the emulsion

As a reference method, emulsion stability was evaluated according to the method of Tornberg and Hermansson (1977). This method is based on a direct determination of fat content of the original and bottom fraction of the centrifuged emulsion. Some modifications were applied to the method taking into account destabilization conditions (centrifugation) used in our studies. Three centrifugal forces (2,000, 4,000 and 12,000 × *g*) and centrifugation for 60 min were used instead of one at 180 × *g* for 15 min. The following equation was used to measure stability index (SI).

$$SI = (F_l/F_o) \times 100$$

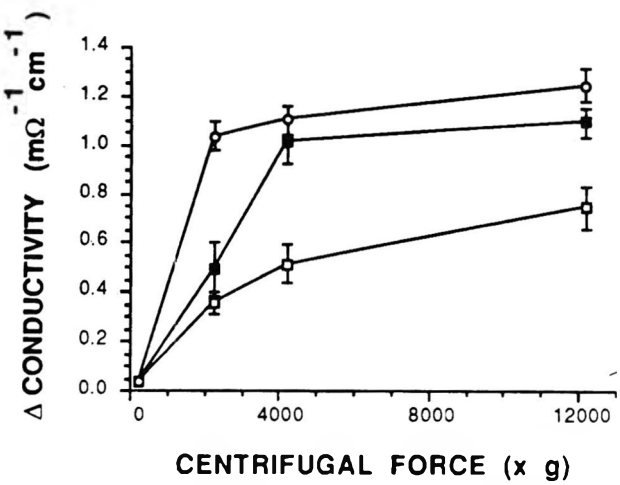

Fig. 2—Effect of centrifugal force on the △ Conductivity of different emulsions: □ trial 1, ■ trial 2, ○ trial 3; See Table 1.

where F_l is the fat percentage of the bottom 50 mL of the centrifuged sample, and F_0 is the initial fat percentage of the original sample. Six determinations were made for each trial. The fat contents, of the whole product and centrifuged fractions, were determined by the Mojonnier fat test (FIL, 1987).

RESULTS & DISCUSSION

THE COMMERCIAL FORMULATION used in our study was microfluidized at various pressures. The microfluidization, a new homogenization process (Paquin and Giasson, 1989), permits high-pressure and considerably reduces diameter of fat globules. This technology was used to control instability because at extremely high-pressures small diameters and large surface area are created. Considering that the same formulation was used, surfactants were not at a sufficient level to properly stabilize newly formed droplets against coalescence, inducing early instability of the products processed at high-pressure. Therefore, emulsions were produced with different stabilities in order to verify the accuracy of the method (Table 1). Trials 1 (19.3 MPa), 2 (48.3 MPa) and 3 (77.2 MPa) represent three products with different stabilities.

Although fat and protein remained constant in each formulation, initial emulsion conductivity (C_o) is important since it characterized the original physical state of the sample. Table 1 presents the results (three repetitions in duplicate) obtained for all original emulsions processed at different pressures. A slight decrease in conductivity was observed with increase in pressure. This can be related to a finer distribution of the smaller diameter fat globules. These values represent more than the fat content obtained with the method of Tornberg and Hermansson (1977). They also reflect a general picture of the total physical state of the emulsion including fat globules, proteins, lactose and minerals. For this reason, stability of the emulsions is obtained by subtracting the conductivity of the centrifuged lower fraction (C_l) from the original conductivity (C_o) in order to render the difference in conductivity (△ Conductivity) more representative of the stability.

An evaluation of milk-based infant formula stability, in terms of △ Conductivity, as a function of centrifugal force for trials 1, 2 and 3 is presented in Fig. 2.

The △ Conductivity increased with product destabilization. According to Kato et al. (1985), the conductivity method is based on the principle that conductivity increases as a function of time due to creaming of fat globules. These globules are nonconductors with respect to the water-protein mixture which, on the other hand, is a good conductor (Webb et al., 1970). Product creaming is caused by centrifugation, hence it is an accelerated aging process (Sherman, 1971), that induces sep-

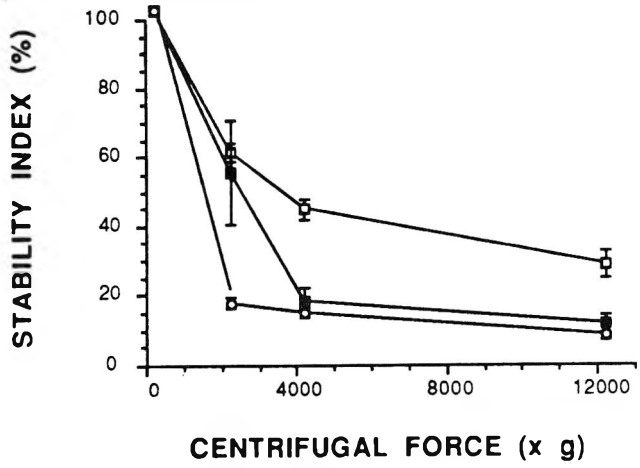

Fig. 3—Effects of centrifugal force on the stability index of different emulsions: □ trial 1, ■ trial 2, ○ trial 3; See Table 1.

aration of the low density oil phase. Increasing the centrifugal force, therefore increases destabilization and, as a result, increases △ Conductivity of the sample.

According to Becher (1965), centrifugal forces of 2,000, 4,000 and 12,000 × *g* applied for 60 min would be equivalent to the effect of gravity for about 3, 6 and 18 months. However, a study by Walstra and Oortwijn (1975), indicated centrifugal creaming, according to Stoke's equation, is higher than gravity creaming. Centrifugation increases the number of collisions between globules and therefore accelerates creaming.

The SI method was used as a reference to determine the accuracy of the conductivity method in estimating the emulsion stability. The stability index (SI), resulting from centrifugal force for trials 1, 2 and 3, is presented in Fig. 3. SI decreases with increase in centrifugal force. A marked difference occurred among trials 1, 2 and 3. In Fig. 2 and 3, results indicate that trials 2 and 3 were less stable than trial 1. At 4,000 × *g* trials 2 and 3 had an SI lower than 30% and a △ Conductivity higher than 1 $m\Omega^{-1}cm^{-1}$. On the other hand, trial 1 samples had an SI value of 65% at 2,000 × *g* corresponding to a △ Conductivity near 0.35 $m\Omega^{-1}$ cm^{-1}. At 12,000 × *g*, trial 1 had a SI higher than 30% representing high stability compared to trials 2 and 3 (20%).

The two methods for evaluating emulsion stability were compared for each centrifugal force used (2,000, 4,000 and 12,000 × *g*). Fig. 4a, b and c present the three trials submitted to three levels of accelerated aging. The stability of trial 1, 2 or 3 progressively changed with each centrifugal force. The correlation coefficients between SI and △ Conductivity were: 0.942 at 2,000 × *g*, 0.960 at 4,000 × *g* and 0.931 at 12,000 × *g*. The slopes of the correlation curves were not the same at each centrifugal force, but △ Conductivity offered a good response to the stability value. It is comparable to the value for SI, as shown by the correlation. These results indicate that with products that differed in stability and with three different accelerated forces (low, medium and high), the conductivity method predicted with as much accuracy as the stability test, the shelf life. It worked faster with simple manipulations.

CONCLUSION

OUR RESULTS demonstrate that emulsion stability can be determined by measuring conductivity combined with centrifugation. The conductivity method can be done in 60 sec, and is easy to carry out, compared to a measurement of water or fat content which takes 20 to 30 min for each sample. Furthermore, conductivity is a direct measure of physical changes and thus gives a more accurate picture of the product state. Compared to the method of Kato et al. (1985), which only works with model emulsions of weak stability, our combined

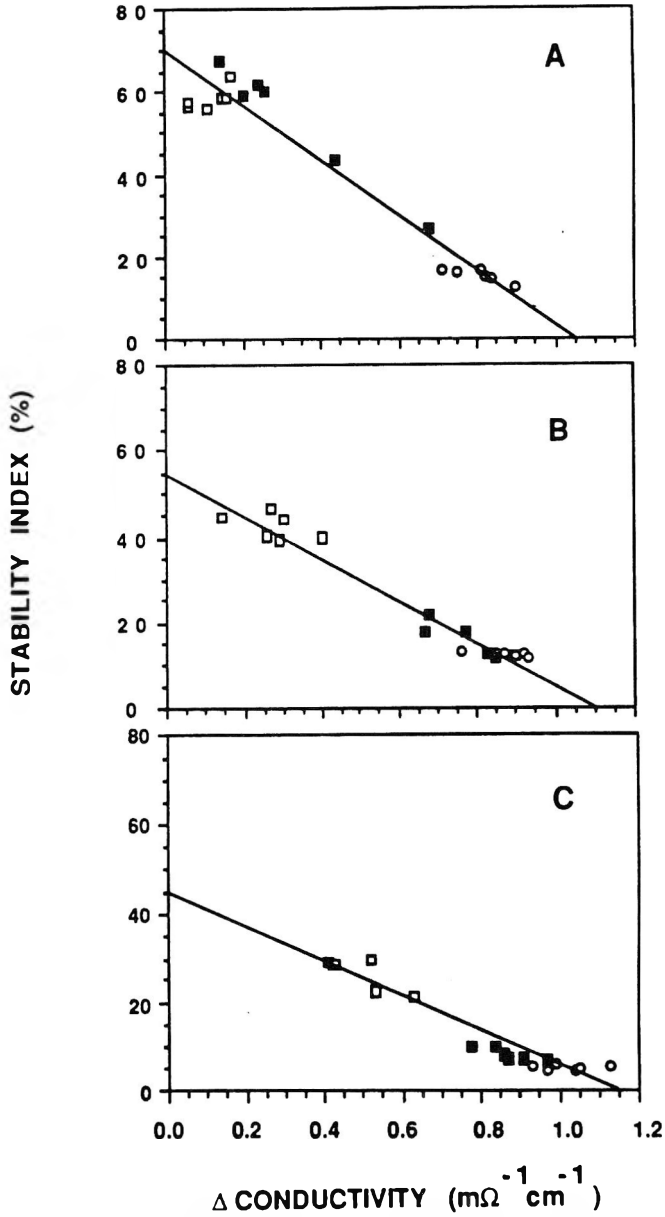

Fig. 4—Relationship between △ Conductivity and stability index with gravitational force: A = 2,000, B = 4,000 and C = 12,000 × g; □ trial 1, ■ trial 2, ○ trial 3; See Table 1.

centrifugation-conductivity method allows one to determine the stability of complex commercial emulsions. This method could be useful for quality control or product development of food emulsions. Other studies are in progress to correlate shelf life and accelerated stability tests.

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Efficiency of Removing Volatiles from Menhaden Oils by Refining, Bleaching, and Deodorization

CHARLES F. LIN, THOMAS C.-Y. HSIEH, JANE B. CROWTHER, and ANTHONY P. BIMBO

ABSTRACT

Atlantic and Gulf of Mexico menhaden (*Brevoortia* spp) oils from refining, bleaching and deodorizing steps were analyzed for volatile components by combined techniques of dynamic headspace sampling, high resolution gas chromatography and mass spectrometry. Results indicated that alkali-refining and clay-bleaching removed many odorous short-chain oxygenated compounds and alkylbenzenes; steam-deodorization (200–208°C, 3 hr) eliminated most volatiles. Some volatiles in deodorized samples were attributed to container material and antioxidants.

INTRODUCTION

RECENT INCREASED public awareness of possible health benefits reducing cardiovascular risks by supplementing diets with n-3 polyunsaturated fatty acids (PUFAs) has created a strong demand for purified fish oil capsules and concentrates (Bimbo, 1987). Direct use of deodorized fish oil for human food consumption in the U.S. has not been approved by Food & Drug Administration (FDA) except the hydrogenated oils which contain little n-3 PUFAs (Cooper, 1989). Currently, there are no commercial scale menhaden oil refineries in the United States. Over 90% of the U.S. crude oil production is exported to Europe and, in turn, imported again as refined fish oil capsules at a cost of almost $150 million annually at the consumers' level (Bimbo, 1987). Once the pending petition of GRAS use of these oils in foods is approved by the U.S. FDA, a potential market may motivate the domestic fish oil industry to engage in refining and deodorization of fish oils. Such processing steps to remove harmful components and undesirable odorous volatiles from the crude oil are necessary for production of acceptable food-grade oils (Stansby, 1973). Hsieh et al. (1989) identified 55 volatile odorous compounds in crude menhaden fish oil by direct dynamic headspace sampling, high resolution gas chromatography and mass spectrometry (DHS/GC/MS).

The objectives of our study were (1) to identify the volatile compounds in menhaden oils after refining, bleaching and deodorization, and (2) to compare efficiency of removing volatile odorous components from these processed oils.

MATERIALS & METHODS

Materials

Menhaden fish were harvested in 1988 from the Gulf of Mexico (*Brevoortia patronus*) and from the Atlantic Ocean (*Brevoortia tyrannus*). Production of crude, bleached, refined and deodorized menhaden oils was carried out by Zapata Haynie Corp. (Reedville, VA). The laboratory scale refining processes used in our study included (1) alkali-refining according to AOCS Method Ca-9a-52 (AOCS, 1980), (2) clay-bleaching using Tonsil Optimum FF(S) activated bleaching clay (L. A. Salomon & Bro., Port Washington, NY), and (3) steam-deodorization using 200–208°C steam, generated from a volume of water equal to 6% of the oil, for 3 hr. Tenox 20A (0.1%, w/w; E. Kodak, Kingsport, TN) and citric acid (0.1%) were added to deodorized oils as antioxidants. The crude oils had strong fishy and oily odors. The deodorized oils had only clean, oily odor. Bleached and refined oils had a strong oily odor with a fishy note similar to the crude oils. The material for containers of the oil samples contained 88% high density polyethylene and 12% Nylon. Authentic standards were obtained from commercial sources.

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Chemical analyses

Free fatty acids, iodine values, peroxide values and anisidine values of menhaden oils were analyzed using AOCS Official Methods (1980). Completely random design (Steel and Torrie, 1980) and Tukey's test were used to determine the significant levels among treatments (SAS Institute, Inc., Cary, NC).

Dynamic headspace sampling (DHS)

Volatile components in fish oils were collected with a dynamic headspace sampler consisting of a Tekmar 4200/4000 (Cincinnati, OH) system. Blank samples were analyzed periodically to ensure no contamination occurred. An aliquot of 0.10g each of the crude, alkali-refined (step I) and alkali-refined/clay-bleached (step II) oil samples, and 0.25g of alkali-refined/clay bleached/steam-deodorized (step III) oil were used separately for each analysis. Each sample was pre-purged at ambient temperature with helium gas (99.999%) at 40 mL/min for 2 min to remove oxygen inside the sample tube. The sample was then heated and maintained at 65°C for 30 min to allow volatile components to be purged and absorbed onto a trap cartridge containing Tenax TA (0.3g, 60/80 mesh, Chrompack Inc., Raritan, NJ). Volatiles trapped in Tenax TA were flash-desorbed at 185°C with a helium flow 40 mL/min, and cryogenically (liquid nitrogen) focused in a fused silica capillary column prior to chromatography.

Gas chromatography/mass spectrometry (GC/MS)

Separation of volatile components was performed on a fused silica capillary column (Supelcowax 10, 60m length × 0.25 mm i.d. × 0.25 μm film thickness) installed in a Hewlett-Packard 5792A GC (Palo Alto, CA). Helium, linear velocity 25 cm/sec, was used as carrier gas. The column temperature program was: 40°C, for 5 min, increased to 175°C at 1°C/min, further increased to 195°C at 5°C/min,

Table 1—Chemical analyses of free fatty acids (FFA), iodine values (IV), peroxide values (PV) and anisidine value (AV) of menhaden oils from successive refining and deodorization

Oil samples	%FFA	IV	PV	AV
Gulf menhaden				
Crude	1.86[a](0.01)	171.1a(1.53)	4.6[a](0.12)	21.0[a](0.50)
Step I[e]	0.15[b](0.01)	167.2[a](1.08)	4.4[b](0.06)	20.5[a](0.44)
Step II[f]	0.14[b](0.00)	169.9[a](1.33)	3.9[c](0.00)	20.1[a](0.27)
Step III[g]	0.1[c](0.00)	169.4[a](1.19)	1.0[d](0.00)	10.5[b](0.50)
Atlantic menhaden				
Crude	1.8[a](0.01)	173.8[a](0.12)	5.2[a](0.01)	19.5[a](0.44)
Step I[e]	0.15[b](0.00)	173.9[a](0.10)	3.3[b](0.08)	19.1[a](0.27)
Step II[f]	0.13[c](0.01)	172.1[a](1.90)	2.2[c](0.03)	19.1[a](0.17)
Step III[g]	0.08[d](0.00)	173.3[a](2.14)	1.5[d](0.03)	10.0[b](0.10)

[a–d] Means of three determinations, standard deviations in parentheses. Data within column bearing different superscripts differ significantly at $p<0.05$. Difference between Gulf and Atlantic menhaden oils was not determined.

[e] obtained by alkali-refining.

[f] obtained by alkali-refining and clay-bleaching.

[g] obtained by alkali-refining, clay-bleaching, and steam-deodorizing.

Table 2—Volatile compounds in the dynamic headspace of menhaden oil samples from refining and deodcrization

		Gulf oils				Atlantic oils			
			Step I	Step II	Step III		Step I	Step II	Step III
Compound name	Ret. time	Crude area %	% of crude	% of crude	% of crude	Crude area %	% of crude	% of crude	% of crude
octane	6.5	1.31	23	53	13	0.24	46	78	3
nonane	8.5	0.71	46	63	0	0.01	62	87	2
decane	13.3	1.02	42	75	0	0.19	34	78	2
undecane	22.4	1.33	34	75	0	0.19	55	84	0
dodecane	35.2	2.12	28	72	0	0.21	105	113	0
tridecane	49.1	2.22	85	170	0	0.27	68	69	0
tetradecane	62.5	1.55	127	127	t	0.51	46	64	0
pentadecane	76.1	8.34	160	112	2	5.71	46	125	t
hexadecane	88.3	0.65	97	41	2	0.65	8	83	3
heptadecane	101.1	12.71	40	2	0	1.03	29	84	0
octadecane	112.5	0.12	0	0	0	0.08	t	1	0
nonadecane	123.6	0	–	–	–	0.04	0	t	0
2-propanol	9.8	0.45	21	70	t	0.05	0	0	14
2-pentanol	24.5	0.09	11	29	0	0.12	20	0	0
1-butanol	28.9	0.22	12	28	t	0.04	0	93	5
1-pentanol	41.8	0.13	22	0	0	0.09	0	15	0
1-hexen-3-ol	42.6	0.91	8	37	0	0.16	51	52	0
(E)-2-penten-1-ol	50.8	0.02	0	0	0	0.03	34	43	0
1-hexanol	56.3	0.94	17	119	0	0.02	223	421	0
2-hexen-1-ol	63.5	0.62	10	4	0	0	–	–	–
1-octen-3-ol	69.4	0.22	119	40	0	0.11	26	103	0
2-nonanol	80.1	0.13	52	25	0	0.01	0	652	0
1-octanol	83.4	0.04	0	0	0	0.04	0	0	0
1,2-propanediol	86.2	0	–	–	0.29*	0	–	–	0.31*
3,5-octadien-2-ol	90.6	0.12	61	34	0	0.04	20	91	0
2-undecanol	103.5	0.22	54	0	0	0.11	0	0	0
1-decanol	106.2	0.31	22	0	0	0.47	6	161	0
1-dodecanol	129.6	t	0	0	0	0.01	0	0	0
phenol	135.4	0	–	–	–	0.02	0	0	0
n-butanal	8.2	0.97	12	47	1	0.18	66	70	3
n-pentanal	12.3	0.82	28	79	t	0.27	70	164	2
(Z)-2-butenal	17.1	0.55	12	63	0	0.13	42	71	0
n-hexanal	21.2	1.56	22	58	2	0.31	119	259	2
(Z)-2-pentenal	26.8	1.48	11	42	0	0.46	60	72	0
n-heptanal	33.6	1.42	15	38	t	0.17	114	210	0
(E)-2-hexenal	37.9	0.97	13	43	0	0.18	98	177	0
(E)-4-heptenal	41.3	0.24	38	160	0	0.06	55	4	0
n-octanal	47.5	0.61	17	58	t	0.13	64	117	0
(Z)-2-heptenal	52.1	0.31	20	54	0	0.14	38	87	0
n-nonanal	61.7	0.51	51	74	0	0.08	84	0	0
(Z,Z)-2,4-hexadienal	63.1	0	–	–	–	0.02	42	185	0
(Z)-2-octenal	63.9	0.13	96	115	0	t	0	0	0
(E)-2-octenal	66.3	0.43	5	33	0	0.11	59	118	0
2,4-heptadienal[b]	71.2	0.69	51	29	0	0.25	65	163	0
n-decanal	74.1	0.15	26	38	0	0.14	44	65	0
2,4-heptadienal[b]	74.8	1.39	54	17	0	0.66	51	110	0
benzaldehyde	78.5	0.34	27	21	5	0.05	82	59	12
(E)-2-nonenal	80.5	0.14	74	46	0	0.05	25	129	0
(Z)-4-decenal	81.5	0.14	62	110	0	0.33	3	84	0
(Z,Z)-2,4-octadienal	83.5	0.11	43	12	0	0.03	0	0	0
2,6-nonadienal[b]	86.6	0.06	133	0	0	0.08	41	141	0
(E,E)-2,4-octadienal	87.1	0.34	24	23	0	0.18	0	0	0
(E)-4-decenal	94.3	0.02	0	0	0	0.11	0	0	0
(Z,Z)-2,6-nonadienal[b]	99.3	0	–	–	–	0.06	0	0	0
(E,E)-2,6-nonadienal[b]	103.1	0	–	–	–	0.06	0	0	0
(Z,Z)-2,6-decadienal[b]	107.4	0.04	0	0	0	0	–	–	–
(E,E)-2,6-decadienal[b]	112.1	0.12	0	0	0	0.02	0	0	0
2,4-undecadienal[b]	113.8	0	–	–	–	0.04	0	0	0
nonatrienal[b]	121.2	0	–	–	–	0.12	0	0	0
nonatrienal[b]	123.2	0.03	0	0	0	0.02	0	0	0
decatrienal[b]	130.4	0	–	–	–	t	t	t	0
decatrienal[b]	133.5	0	–	–	–	t	t	t	0
2-propanone	5.3	0	–	–	18.11*	0	–	–	5.34*
2-butanone	6.9	0.64	9	51	0	0.12	0	33	0
1-penten-3-one	15.3	0.95	21	51	0	0.36	66	72	0
3-hexanone	17.6	0.38	11	80	0	0.07	138	257	11
2,3-pentanedione	19.1	0.25	11	37	0	0.14	14	40	0
2,3-hexanedione	27.3	0.05	95	415	0	0	–	–	–
3-heptanone	30.8	2.77	18	37	0	0.87	89	73	0
3-nonanone	52.9	0	–	–	2.35*	0	–	–	0.1*
3-methyl-2,4-pentanedione	61.1	0.23	14	41	0	0.06	31	135	0
3-nonen-2-one	77.9	0.22	62	124	1	0.15	63	141	1
(E,E)-3,5-octadien-2-one	84.5	0.08	21	47	0	0.06	49	120	0
acetophenone	95.2	0.21	56	31	0	0.11	33	75	0
2-ethylcyclopentanone	105.6	0.08	0	0	0	0	–	–	–

Table 2—Continued

Compound name	Ret. time	Gulf oils				Atlantic oils			
			Step I	Step II	Step III		Step I	Step II	Step III
		Crude area %	% of crude	% of crude	% of crude	Crude area %	% of crude	% of crude	% of crude
benzene	10.4	0.46	21	34	3	0.07	26	20	16
toluene	16.8	0.28	18	62	4	0.06	54	0	5
ethylbenzene	25.8	0.06	34	191	0	0.18	3	0	0
p-xylene	26.1	0.01	16	288	0	0.03	20	297	0
m-xylene	27.8	0.21	27	40	0	0.13	0	14	0
o-xylene	33.2	0.11	28	174	0	0.11	17	33	0
propylbenzene	36.1	0.15	49	45	0	0.01	0	152	0
C3-alkyl benzene	38.2	0.18	0	0	0	0.11	0	18	0
4-ethyltoluene	38.9	0.22	118	63	0	0.11	15	0	0
1,3,5-trimethylbenzene	41.1	0.21	0	0	0	0.07	0	261	0
2-ethyltoluene	44.1	0.89	36	79	0	0.21	138	195	0
4-isopropyltoluene	45.6	0.31	33	92	0	0.08	132	214	0
1,2,4-trimethylbenzene	46.1	0.24	10	21	0	0.15	0	23	0
3-isopropyltoluene	49.5	2.36	22	50	0	0.08	29	24	0
1,4-diethylbenzene	50.1	0	–	–	–	0.02	377	473	0
C3-alkyl benzene	52.7	0.07	72	244	0	0.01	0	0	0
1,2-diethylbenzene	53.3	0.04	125	402	0	0.06	0	0	0
1,2,3-trimethylbenzene	53.9	0.23	22	20	0	0.04	74	193	0
2-ethyl-1,4-dimethylbenzene	57.9	0	–	–	–	0.14	36	18	0
C5-alkyl benzene	59.1	0.11	0	0	0	t	0	0	0
C4-alkyl benzene	60.2	0.11	0	0	0	0.04	0	0	0
1,2,4,5-tetramethylbenzene	66.1	0.01	119	68	0	0.02	0	0	0
1,4-dichlorobenzene	68.1	0.34	66	64	68	0.11	45	38	35
C4-alkyl benzene	73.7	0.09	0	0	0	0.04	0	0	0
1,2,3,4-tetramethylbenzene	75.3	0.04	86	9	0	0.09	8	0	0
2-ethylfuran	10.9	3.95	23	34	t	1.21	52	84	t
chloroform	12.9	0	–	–	0.43*	0	–	–	0.61*
dimethyl disulfide	20.2	0.23	14	0	0	0.05	949	400	0
limonene	34.5	0.18	0	0	0	0.14	24	42	0
2-pentylfuran	39.8	0.41	11	46	0	0.06	114	135	0

[a] Step I = Alkali-refined; Step II = Alkali-refined/clay-bleached; Step III = Alkali-refined/clay-bleached/steam-deodorized with 0.1% Tenox 20A.
[b] Configuration of geometric isomers not determined.
* Number represents % of total in Step III sample.
– Compounds are not detected in samples.
% = area/area of crude oil × 100.
t = trace amount present (< 1%).

maintained at 195°C until the end of the GC run. Total run time was 140 min. The MS procedure has been described elsewhere (Hsieh et al., 1989).

RESULTS & DISCUSSION

CHEMICAL ANALYSIS of free fatty acids, iodine values, peroxide values and anisidine values of crude, and oil samples sequentially processed by alkali-refining, clay-bleaching, and steam-deodorization from Gulf and Atlantic crude oils are shown in Table 1. Alkali-refining significantly ($p < 0.05$) removed almost 90% of the free fatty acids from the crude oils. Subsequent clay-bleaching reduced a small but significant ($p < 0.05$) amount of free fatty acids from the Atlantic sample. Little change was observed in iodine values of oil samples within either sample groups ($p > 0.05$) (Table 1). This was consistent with the fact that alkali-refining, clay-bleaching, and steam-deodorization do not, in general, destroy double bonds in oil samples. The peroxide values were effectively reduced ($p < 0.05$) to 20–30% of original levels in the crude samples by successive alkali-refining, clay-bleaching and steam-deodorization. These three refining steps can be used to improve the peroxide value. Anisidine value is a measure of the alpha-beta unsaturated aldehydes. Alkali-refining and clay-bleaching did not significantly ($p > 0.05$) change anisidine values, while steam-deodorization reduced by about 50%, the original alpha-beta unsaturated aldehydes in the oils ($p < 0.05$) (Table 1).

Since proximate chemical analyses cannot provide specific information about individual odorous compounds in these samples, combined techniques of DHS/GC/MS were used for further analysis. The volatile components identified in the oil samples from different steps of refining are listed in Table 2. These are probably by-products from degradation of lipids, proteins and amino acids by microbial spoilage, lipid autoxidation or by lipoxygenase-catalyzed reactions (Stansby, 1971, 1973; St. Angelo et al., 1980; Frankel, 1982).

A series of alkanals, alkenals, alkadienals, and alkatrienals were in both Gulf and Atlantic crude menhaden oils (Table 2). These aldehydes, contributing to major odors in oils, were used as chemical markers for monitoring efficiency of removing volatiles from oil by alkali-refining, clay-bleaching and steam-deodorizing. Saturated aldehydes (C4–C10) were detected in crude menhaden oils and were responsible for the characteristic oxidized oil odors, such as green grassy, waxy, and rancid oily. After steam-deodorization, saturated aldehydes (C4–C7) were detected at less than 4% of the levels in the crude oils. None of the other saturated aldehydes were in the steam-deodorized oils.

Unsaturated aldehydes (C4–C10) were found in the crude, alkaline-refined and clay-bleached but not steam-deodorized oils. Among these unsaturated aldehydes, (Z)-4-heptenal was reported to have oxidation flavors when higher than 15 ppb (Badings, 1965, 1973).

Alkadienals, 2,4-hexadienal, 2,4-heptadienal, 2,4-octadienal and 2,6-nonadienal, were found in crude, alkali-refined and clay-bleached, but not in deodorized menhaden oils. On the other hand, 2,4-decadienal and 2,4-undecadienal were only in the crude oils. These aldehydes are believed to result either from autoxidative or enzymatic oxidation of n-3 polyunsaturated fatty acids. Under abusive or poor storage conditions, they would undergo further hydroperoxidation (Frankel, 1982) and/or retro-aldol degradation (Josephson and Lindsay, 1987) to produce shorter-chain volatile derivatives.

Alkatrienals, such as nonatrienals and decatrienals, also were found at ppb levels in dynamic headspace of the crude oils. This was consistent with findings of Hsieh et al. (1989) that several alkatrienals gave oxidized fish oil notes in crude Atlantic menhaden oil. Meijboom and Stroink (1972) reported that (E,Z,Z)-2,4,7-decatrienal caused a fish or whale oil flavor in a strongly autoxidized fish oil.

Almost 100% of most odorous unsaturated aldehydes, and

80% of the unpleasant saturated aldehydes, in the crude oils could be removed by alkali-refining and clay-bleaching (Table 2). However, the additional step of steam-deodorization was necessary to remove a total of 96–99% of most aldehydes from the dynamic headspace of the oils. Additional analyses on the antioxidant Tenox 20A alone (data not shown) suggested that some of the trace (ppb or lower levels) headspace volatiles in the deodorized oils might have been introduced through the Tenox 20A addition.

Several alcohols, with a saturated chain from C3 to C12 and an unsaturated chain such as 1-hexen-3-ol, (E)-2-penten-1-ol, 2-hexen-1-ol, 1-octen-3-ol and 3,5-octadien-2-ol, were in the dynamic headspace of the crude oils. The combination of alkali-refining and clay-bleaching methods effectively reduced the volatile alcohols up to 96% in crude oils. After alkali-refining, 1-dodecanol and phenol were completely removed, and 1-decanol was removed by clay-bleaching. Except 2-propanol and 1-butanol, all of the alcohols in crude oils were removed by steam-deodorization. However, 1-butanol and 1,2-propanediol (ppb level) were detected in dynamic headspace of Tenox 20A which most probably contributed these volatiles. These volatiles were not detected in oils from prior steps.

A total of eleven ketones with different chain lengths were detected in crude menhaden oils (Table 2). Alkali-refining removed 100% of 2-ethylcyclopentanone and 40–90% of other ketones. Again, steam-deodorization was very effective in removal of ketones. However, three saturated ketones, 2-propanone (18% and 5%), 3-hexanone (0% and 11%) and 3-nonanone (2% and <1%) in the Gulf and Atlantic menhaden oils, were found after steam-deodorization. Since 2-propanone and 3-nonanone also were found in Tenox 20A, these two may have been added to the oils through Tenox 20A.

Twenty benzene-containing compounds, suspected of environmental origin, were detected in crude Gulf and Atlantic menhaden oils (Table 2). This series of compounds, including benzene, alkyl benzenes, benzaldehyde and 1,4-dichlorobenzene, are strong odor contributing components, and also were used as odor quality markers. Alkali-refining and clay-bleaching removed only part of benzene-containing compounds from crude menhaden oils. Although steam-deodorization removed a large portion of benzene-containing compounds, benzene, toluene, benzaldehyde and 1,4-dichlorobenzene were still found at various levels (Table 2).

Alkanes (C8-C18), benzaldehyde, 1,4-dimethylbenzene, o-xylene, 1-ethyl-2-methylbenzene, 1,4-dichlorobenzene, and phenol were found in the dynamic headspace of the plastic container material (data not shown). Since all oils were packed in plastic containers, some of these volatile compounds might have migrated from the container to the oils.

Major variations were observed in efficiencies of removal of odorous volatiles by alkali-refining and clay-bleaching. Steam-deodorization was most effective in removing most volatiles. Although the deodorized oils did not give appreciable odors other than that of bland oil by subjective evaluation, many of the volatile compounds were still detected by DHS/GC/MS at ppb levels or lower. Choice of containers and antioxidant additives must be carefully considered so that undesirable volatile compounds are not introduced. In addition to conventional chemical analyses and sensory evaluation, monitoring of odorous volatiles by DHS/GC/MS can enhance quality assurance in the fish oil industry to produce food grade fish oils with minimum odor and volatile compounds.

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Prediction of Levels of Cholesterol Oxides in Heated Tallow by Dielectric Measurement

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ABSTRACT

Two quality control procedures currently used for frying oil were evaluated with respect to efficiency of prediction of cholesterol oxidation products (COPS) levels. Tests in a French fried potato model system (90% tallow/10% cottonseed oil) showed the correlation between dielectric measurement and the COPS levels in the frying medium was highly significant ($r = 0.94$, $N = 30$, $P < 0.01$). Free fatty acid "test strips" showed no quantifiable change during 112 hr and, therefore there was no correlation with COPS levels. Free fatty acids (AOAC procedure 28.032 [b]) highly correlated with COPS levels ($r = 0.94$, $N = 30$, $P < 0.01$).

INTRODUCTION

AUTOXIDATION has been shown to take place in air-aged or colloid-dispersed cholesterol. However, not until development of modern analytical methods such as capillary gas chromatography (GC) have cholesterol oxidation products (COPS) been clearly demonstrated in food (Addis, 1986; Addis and Park, 1989).

Recently, COPS have gained extensive attention as a result of their relation to potential deleterious health problems which include atherogenicity, cytotoxicity, mutagenicity and possible carcinogenicity (Smith, 1981; Addis et al., 1983; Addis and Park, 1989). Accordingly, the scientific study of autoxidation of cholesterol in food has become a critical research need. Previous studies have led to development of chromatographic methods for quantification of COPS in foods using high performance liquid chromatographic (HPLC) and capillary GC techniques (Park and Addis, 1985a, b). These methods demonstrated, high levels of COPS in some dehydrated food products (Addis and Park, 1989; Park and Addis, 1987; Park and Addis, 1985a).

Heated tallow displayed four COPS (7α-hydroxy-, 7β-hydroxy- and 7-ketocholesterol and cholesterol α-epoxide) identified by capillary GC-mass spectrometry (MS) by Park and Addis (1986a, b). The objective of our study was to evaluate quality control methodology which could be employed during frying operations to reduce lipid oxidation, thereby improving sensory quality and reducing exposure to COPS by fast-food patrons.

MATERIALS & METHODS

Sample preparation

To 0.1–0.12g shortening, 50 μg 5α-cholestane were added as an internal standard for GC analysis. Dried nonsaponifiables obtained were subjected to trimethylsilylation according to Park and Addis (1985a, b) except that nonsaponifiables were washed with 5 mL 0.5N KOH once and then with distilled water twice.

GC quantitation of COPS

The GC analyses of nonsaponifiables were made with a Varian Vista 6000 GC equipped with a flame ionization detector for the quantitation of COPS as trimethylsilyl (TMS) ether sterols. The details of this method have been described by Park and Addis (1986a,b).

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Peak identification

Peak identification was done by (1) co-chromatography of the suspected peak from the sample with the corresponding standard by comparing respective retention times and (2) MS, using a Kratos MS-25 GC/MS with a DS-55 data system (Kratos, Ramsey, NJ). Ion source temperature was 220°C and electron impact ionization was 70 e.V. Spectra of suspected peaks were compared to known standards.

Peroxides and free fatty acids

Peroxide values (PV) and free fatty acids (FFA) were determined according to AOAC procedure 28.025 and AOAC procedure 28.032(b), respectively.

Food oil sensor

Food oil sensor (FOS) model N1-21A (Northern Instruments Corp., Lino Lakes, MN) was used to measure the change in dielectric properties in heated tallow relative to fresh tallow. The instrument was calibrated with fresh shortening of the same type as used in frying. FOS readings increase as triglycerides hydrolyze to free fatty acids and glycerol, both products being more polar than the original triglycerides. Polarity also increases as glycerides, cholesterol and free fatty acids undergo autoxidation.

Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined by a slight modification of the method of Rethwill et al. (1981) in which tallow (4.000g) was homogenized in 10% trichloroacetic acid-1M H_3PO_4 and centrifuged 20 min at 1000 × g.

Shortening monitor kit

The shortening monitor kit test strips (FFA-TS) manufactured by 3M Company (St. Paul, MN) were designed to monitor shortening breakdown by measuring free fatty acid formation. These strips were compared to the AOAC procedure for FFA determination and levels of lipid oxidation products. Briefly, FFA-TS were dipped into hot shortening. Changes in appearance indicated FFA. The number of blue bands which changed into yellow bands roughly quantified the degree of FFA formation. The FFA-TS were tested in heated vegetable shortening, as a positive control, and were found to change color.

Sample collection and preparation

Frying shortening employed was a mixture of 90% refined, deodorized tallow with 10% commercial cottonseed oil. Fresh potatoes were purchased from a local food store, and potato strips were made using a food processor. The potato strips were washed with hot water at 70–80°C twice, drained and frozen at −32°C for no longer than 10 days. Mixed shortening (3.33 kg) was placed into a household fryer (Model DCP-6, Dazey Corporation, Industrial Airport, KS) and potato strips (100g) were fried each hour for 5-8 min at 168 ± 10°C for 16 hr each day. The oil was filtered at the end of each day through Spectrum Laboratory filter paper (Curtin Scientific Company, Houston, TX), and 600-800 mL of mixed fresh shortening were added to keep the oil at a constant level. The shortening was cooled overnight and preheated 30 min before frying each day.

Shortening samples (160 mL) were removed at 8 hr intervals (twice

Table 1—Values for free fatty acids, polarity, autoxidation and cholesterol oxidation products in heated oil (90% tallow/10% cottonseed oil) as affected by frying time

Time	Measurement[a,b,c]					
(hr)	FFA-TS	FFA-AOAC	FOS	PV	TBARS	COPS
0	0.0	0.03	0.03	0.11	0.27	4
8	0.0	0.04	0.27	0.22	0.33	6
16	0.0	0.07	0.44	1.51	0.37	24
24	0.0	0.09	0.44	1.52	0.40	25
32	0.0	0.15	0.84	2.92	0.51	31
40	0.0	0.18	1.44	2.16	0.40	44
48	0.0	0.23	1.24	2.65	0.71	54
56	0.0	0.29	1.39	2.38	0.68	46
64	0.0	0.36	1.89	2.42	0.75	50
72	0.0	0.42	1.97	3.24	0.69	48
80	0.0	0.54	2.14	3.46	0.73	56
88	0.0	0.62	2.36	2.55	0.36	66
96	0.0	0.78	2.54	1.19	0.49	64
104	0.0	0.91	2.82	1.41	0.46	78
112	0.0	1.27	3.03	2.05	0.50	113

[a] Mean of duplicate determinations except for COPS (N = 4).

[b] Symbols used: FFA-TS, free fatty acid test strips (Shortening Monitor Kit); FFA-AOAC, free fatty acids — Association of Official Analytical Chemists; FOS, food oil sensor; PV, peroxide value; TBARS, thiobarbituric acid reactive substances; COPS, cholesterol oxidation products.

[c] Units for measurements: FFA-TS, arbitrary color scale; FFA-AOAC, % FFA as oleic; FOS, arbitrary scale; PV, meq/kg; TBARS, ppm; COPS, ppm.

Table 2—Correlation matrix among methods of oil evaluation[a]

	Measurements[a]			
	COPS	FOS	FFA	TBARS
COPS	—	—	—	—
FOS	0.94[b]**	—	—	—
FFA	0.94**	0.93**	—	—
TBARS	0.35	0.39*	0.19	—
PV	0.42	0.43*	0.19	0.74

[a] Symbols used: FFA-AOAC, free fatty acids — Association of Official Analytical Chemists; FOS, food oil sensor; TBARS, thiobarbituric acid reactive substances; COPS, cholesterol oxidation products.

[b] Simple correlation coefficient (n = 30)

*$p < 0.05$

**$p < 0.01$.

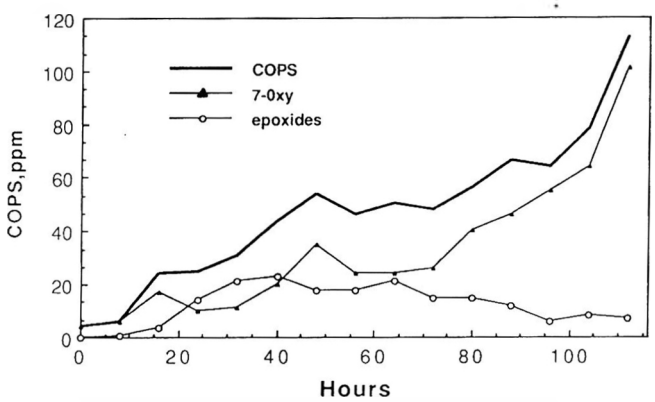

Fig. 1—Cholesterol oxidation products vs frying time. Symbols used in the figure: ▲———▲ 7-oxy = 7α-hydroxycholesterol + 7β-hydroxycholesterol + 7-ketocholesterol; ○———○ epoxides = α-epoxide + β-epoxide. ——— COPS = total cholesterol oxidation products.

each day), 20 min after fries were removed from the oil. FOS dielectric measurement, FFA (AOAC and FFA-TS), and PV were determined. The remaining oil sample was placed in a 15 mL glass vial, flushed with N_2 for about 10-15 min, capped, wrapped with aluminum foil and held at −30°C for not longer than 20 days until COPS could be analyzed.

RESULTS & DISCUSSION

WHEN FRIES were prepared in a simulated restaurant system, COPS increased with frying time (Fig. 1). The 7-oxy fraction and total COPS increased in a fairly steady manner with heating time whereas total epoxides appeared to reach a broad maximum from 40 to 65 days and gradually decline thereafter. These results were fairly consistent with those of Park and Addis (1986a, b). In both our present study and in the report of Park and Addis (1986a), epoxide levels reached a maximum and then declined while 7-ketocholestero. continued to increase. Park and Addis (1986b) heated tallow for 70, 144 and 216 hr at 135, 150, 165 and 180°C. α-Epoxide was detected at 135, 150 and 165°C for all three time periods. However, α-epoxide could not be detected at 180°C for any of the three time periods. Although α-epoxide is known to hydrolyze to 5α-cholestane-3β,5,6β-triol (triol) in the presence of acid (Smith, 1981), this was clearly not the case because no triol could be confirmed by MS. Further studies will be necessary to determine the fate of α-epoxide subjected to high temperature treatment in heated tallow.

Table 3—Cholesterol oxidation products content in frying medium of tallow/cottonseed oil (90/10)

Time	COPS, ppm[a,b]							
(hr)	7α	βep	αep	7β	triol	7k	25-OH	total
0	4	—	—	—	—	—	—	4
8	4	—	—	1	—	-	—	6
16	14	2	3	1	2	2	—	24
24	4	3	11	1	1	5	4	27
32	2	4	17	—	2	5	1	31
40	3	9	14	1	1	16	4	44
48	3[c]	5	13[c]	2[c]	1	30[c]	—	54
56	7[c]	7	11	3[c]	2	14[c]	2	46
64	3	12	9	3	1	18	4	50
72	3	6	9	3	2	20	5	48
80	6	6	9	9	1	25	2	56
88	3	6	6	14	3	29	5	66
96	3	5	9	23	2	29	1	64
104	6	7	1	31	4	27	2	78
112	6	5	2	42[c]	3	53[c]	2	113

[a] Mean value of n = 4

[b] Symbols used in table: COPS, cholesterol oxidation products; 7α, 7β = 7α- and 7β-hydroxycholesterol; βep, αep = β-epoxide and α-epoxide Triol = cholestane-triol; 7k = 7-ketocholesterol; 25OH = 25-hydroxycholestarol; - = not detected.

[c] Confirmed by mass spectroscopy

Results of the mean values for FFA-TS, FFA-AOAC, FOS, PV, TBARS, and COPS are shown in Table 1 and the correlation coefficients among these measurements are presented in Table 2. The FFA-TS method did not detect changes in FFA throughout the 112 hr heating time. In contrast, COPS, FOS and FFA-AOAC displayed nearly linear, continuous increases with time. The lack of change in the FFA-TS method in spite of a significant rise in FFA-AOAC suggests a serious lack of sensitivity in the FFA-TS method. This finding has been confirmed in discussions with representatives of the fast food industry. If a fast-food restaurant operator relied on FFA-TS to ascertain oil quality, chances are some of the French-fries served would contain high levels of COPS and other potentially undesirable lipid autoxidation products. Indeed, a recent survey we conducted has confirmed high levels of COPS in French-fries from fast-food restaurants (Zhang et al., 1990). Park and Addis (1985) also reported COPS in French fries. TBARS and PV showed the expected increase in values followed by pronounced declines after 80 days of heating. Therefore, PV and TBARS were useful only during the first 80 days.

FOS and AOAC-FFA showed the highest correlation with COPS ($r = 0.94$ $P<0.01$). However, the FOS test was easier and faster than the FFA method, thus making it more appropriate for use in a fast-food restaurant. During 112 hr frying FFA-TS did not detect any COPS changes in the oil which could be quantified on the subjective scale provided. The method therefore lacked sensitivity to reveal changes in FFA although the AOAC-FFA method revealed significant changes. The low correlation coefficient between COPS and TBARS ($r = 0.35$ in Table 3) was clearly an indication of the non-linear rela-

tionship between TBARS and time. TBARS reached a maximum at 64 hr heating, then declined. PV showed a similar trend. Calculation of the correlation coefficient for the first 64 hr between COPS and TBARS revealed a highly significant relationship ($r = 0.87$, $P<0.01$).

It appears that FOS dielectric measurement reflects primarily the increased polarity in heated oil as FFA and other polar compounds are formed and not the slight increase in polarity caused by cholesterol oxidation. The highly significant correlation between FOS and FFA-AOAC ($r = 0.93$, <0.01) supports the concept that the polarity measurement by FOS is closely related to FFA production and perhaps only indirectly correlated with COPS. However, a recent study suggested a chemical mechanism whereby increased FFA accelerated cholesterol oxidation (Kim et al., 1989). FFA and COPS are therefore, possibly correlated because of an underlying chemical mechanism. Further studies are needed on this question. COPS levels correlated highly significantly with AOAC-FFA ($r = 0.94$, $P<0.01$).

The six COPS studied showed different patterns of occurrence during 112 hr frying (Table 3). 7α-Hydroxycholesterol remained at relatively low levels (3 ppm to 14 ppm). 7β-Hydroxycholesterol and 7-ketocholesterol increased with frying oil usage from 0 to 42 and 53 ppm respectively. α-Epoxide reached 17 ppm at 32 hr. and slowly declined thereafter. β-Epoxide reached a maximum at 12 ppm at 64 hr and declined thereafter. It is important to emphasize that there was no doubt concerning existence of COPS based on MS confirmations obtained (Table 3).

The results of this study are significant, demonstrating that a popular quality control procedure (FFA-TS) is useless for prediction of FFA and COPS levels if a tallow/cottonseed (90/10) blend is used. In contrast, the FOS device performed well with respect to correlation with COPS levels and other measures of oil quality. The findings that FOS correlated well with oil quality measurements agreed with results of Fritsch et al. (1979) and Croon et al. (1986).

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Inhibition of Growth and Germination of *C. botulinum* 33A, 40B, and 1623E by Essential Oil of Spices

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ABSTRACT

Effects of clove, thyme, black pepper, pimenta, origanum, garlic, onion, and cinnamon oils on growth and germination of *Clostridium botulinum* types 33A, 40B, and 1623E were studied. At 200 ppm, all oils highly inhibited growth of *C. botulinum* 33A, 40B, and 1623E. At 10 ppm, inhibitory activity of most oils diminished. By activity on *C. botulinum* growth, oils could be divided into three categories: (1) very active: cinnamon, origanum, and clove; (2) active: pimenta, and thyme; (3) least active: garlic, onion, and black pepper. Effectiveness on germination was quite different. At 150 and 200 ppm all oils totally prevented germination. At 10 ppm garlic and onion showed higher activity than the others. Spores of 33A were more sensitive than 40B and 1623E.

INTRODUCTION

SPICES and their derivatives have been used in food preservation over 5000 years. Inhibitory action of spices and their extracts on different microorganisms has been reported (Azzouz and Bullerman, 1982; Conner and Beuchat, 1984: Ueda et al., 1982). Renewed interest in use of spices as antibacterial agents in foods is attributed to two reasons: (1) safety of some food additives is questioned; therefore, there is a trend toward use of natural plant substances; (2) the reduction of salt and sugar in foods for dietary reasons tends to enhance use of seasonings. Most essential oils of spices are classified as GRAS and considered to contain the antimicrobial activity of spices. Limited studies have been carried out on effect of spices and their derivatives on anaerobic microorganisms including *C. botulinum* types (Hall and Maurer, 1986; DeWit et al., 1979; Huhtanen, 1980).

The objective of our investigation was to determine the effects of clove, thyme, black pepper, pimenta, origanum, garlic, onion, and cinnamon oils on growth and germination of *C. botulinum* 33A, 40B, and 1623E in microbiological media.

MATERIALS & METHODS

Essential oils of spices

Essential oils of clove, thyme, black pepper, pimenta, origanum, garlic, onion, and cinnamon were obtained from Fritzsche, Dodge & Olcott Inc. (New York, NY) and stored at 4°C. Appropriate dilutions were prepared in 95% filter-sterlized ethanol on the day of use.

Preparation of *C. botulinum* spores

Spores of *C. botulinum* 33A, and 40B (proteolytic type) were produced by the biphasic culture method of Anellis et al. (1972), whereas spores of 1623E were prepared using the biphasic method of Bruch et al. (1968). The spores were harvested by centrifugation (Rowley and Feeherry, 1970). The harvested spores were suspended in an enzyme solution composed of 100 μg/mL trypsin and 200 μg/mL lysozyme in 0.1M phosphate buffer pH 7.0 (Hawirko et al., 1976). The suspension was incubated 4 hr at 37°C with continuous agitation. The suspension was centrifuged three times 1000 × *g* for 20 min, 4000 × *g* for 10 min, and 10,000 × *g* for 5 min to remove cell debris. The cleaned spores were suspended in sterile distilled water, and adjusted to a concentration such that on addition of 0.2 mL of suspension to 10 mL distilled water the optical density at 600 nm was 0.3. The final spore suspension was stored in 2 mL vials at −20°C. Number of viable spores in the vials was determined by roll tube method (Pierson et al., 1974).

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Effect of spice oils on the growth of C. botulinum

Prereduced peptone yeast extract agar was prepared and dispensed (9.8 mL) into roll tubes (18 × 142 mm; Bellco glass) using the VPI anaerobic system (Holdeman et al., 1977). The prereduced medium was autoclaved at 121°C for 15 min and maintained at 55–60°C in a liquid state. The spice oils at concentrations of 10, 50, 100, 150, and 200 ppm were added to the prereduced medium. Tubes containing various concentrations of oils were inoculated with activated spores (300 spores/tube). Those of type A and B were activated at 80°C for 15 min, while spores of type E were activated at 60°C for 10 min. The inoculated tubes were centrifuged to solidify the medium around the tubes, and incubated at 35°C for 3 days. The number of colonies in the tubes was counted using Darkfield Quebec Colony Counter (American Optical Corporation). The percent growth inhibition (colony formation) caused by each concentration of the oils was determined according to the following equation:

$$\%\text{Growth Inhibition} = \left[1 - \frac{\#\text{ of colonies in treatment tube}}{\#\text{ of colonies in control tube}}\right]$$

The control tube was prepared as described above except that the tube contained no essential oils.

Effect of spice oils on germination

Effect of the spice oils on germination was studied using the microculture method (Duncan and Foster, 1968). A medium of 1% thiotone (BBL, Cockeysville, MD), 1% yeast extract (BBL), 0.4% glucose, and 1.5% agar in 0.05M sodium phosphate buffer pH 7.0 was prepared. The medium was dispensed (9.7 mL per tube) into tubes, autoclaved at 121°C for 15 min and maintained in a liquid state at 55–60°C until used. Filter-sterilized sodium carbonate solution was added to 30 mM and chloramphenicol was added to the medium to a concentration of 10μg/mL. (Sodium carbonate enhances germination rate, whereas chloramphenicol prevents outgrowth of germinated spores, 0.1 mL of appropriate dilution of spice oil was added to different tubes to make final spice oil concentrations of 10, 50, 100, 150, and 200 ppm in the media. About 0.5 ml of agar medium, mixed with spiced oils, was layered on a sterile glass slide (75 × 25 mm). About 20 μL of heat shocked spores (at 80°C for 15 min for types A and B and at 60°C for 10 min for type E) was smeared onto the surface of a sterile glass cover-slip (18 × 18 mm). The spores were allowed to dry on the surface of the cover-slip. The cover-slip was inverted onto the hardened agar surface on the glass slide. The edges of the cover-slip were sealed with vasper. The microcultures were incubated at 32°C for 6 hr, and microcultures were examined under phase contrast microscopy. The germinated spores appeared dark, whereas ungerminated spores appeared bright refractile. The number of germinated and ungerminated spores in ten microscopic fields (MF) were counted. The percent of germination was determined according to the following equations:

$$\%\text{ Germinated spores} = \frac{\#\text{ of germinated spores in 10 MF}}{\text{Total \# of spores in 10 MF}}$$

$$\%\text{ germination} = \frac{X}{Y} \times 100$$

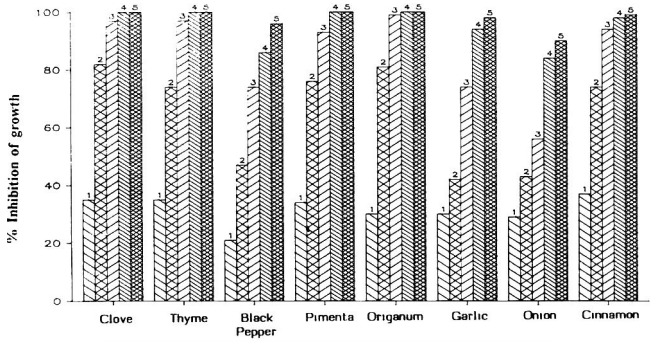

Fig. 1—Growth inhibition of C. botulinum *33A by spice oils in Peptone Yeast Extract Agar media. 1, 2, 3, 4, and 5 on the bars represent oil concentrations of 10, 50, 100, 150, and 200 ppm, respectively.*

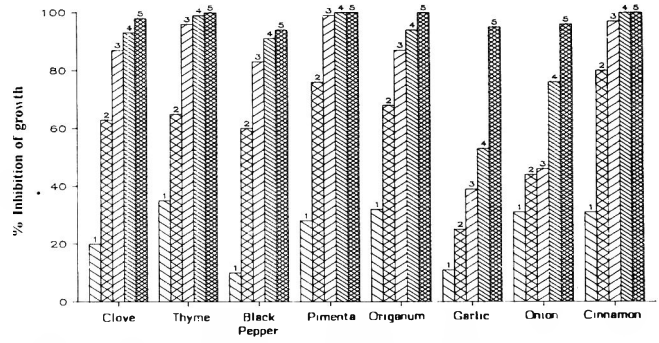

Fig. 2—Growth inhibition of C. botulinum *40B by spice oils in Peptone Yeast Extract Agar media. Legend same as Fig. 1.*

where X refers to % germinated spores in microcultures prepared with a certain type and concentration of oil, and Y represents %germinated spores in microcultures with no spice oil (control).

RESULTS & DISCUSSION

THE EFFECTS of spice oils on the growth of *C. botulinum* 33A are presented in Fig. 1. At 150 and 200 ppm clove, thyme, pimenta, origanum, and cinnamon oils caused 100% inhibition. Onion, garlic, and black pepper oils at the same concentrations caused 86-96% inhibition. At 10 ppm, inhibition ranged from 21% for black pepper to 37% for cinnamon. Ethanol (0.1 mL of 95% in 10 mL medium, used to dilute the essential oils) caused inhibition of 13%. The difference between the growth inhibition at 10 ppm and 0.95% ethanol indicated a slight growth inhibition by the oils.

The pimenta and cinnamon oils were very effective in preventing colony formation by 40B (Fig. 2). These two oils at ≥100 ppm caused over 95% inhibition. As with type 33A, the garlic, onion, and black pepper oils were least effective. These oils allowed a slight growth even at 200 ppm. At 10 ppm, black pepper and garlic caused 10% inhibition, while other oils caused higher inhibition (20–30%).

Spice oils were also effective in controlling growth of type 1623E (Fig. 3). Origanum oil was most effective, causing 98% inhibition at ≥100 ppm. Pimenta, clove, thyme and cinnamon, respectively, followed origanum in their effects. At 10 ppm, the oils caused 4–13% growth inhibition, lower than that of oils on strains 33A and 40B. Ethanol caused inhibition of 9% which is about that caused by oils at 10 ppm.

Overall, all spice oils at 200 ppm were very effective in controlling growth of the spores (300 per roll tube) and there were no significant differences between the oils ($p > 0.05$). At 150 ppm garlic, onion, and black pepper had significantly ($p \leq 0.05$) less effects than the others. At 100 and 50 ppm origanum and clove had significantly higher effect than the others. Oils of garlic, onion, and black pepper produced the least growth inhibition ($p \leq 0.05$). At 10 ppm all oils had very low effects on growth and there were no significant differences between them. The inhibitory concentration of the oils on *C. botulinum* was comparable with the inhibitory effect on other microorganisms, e. g. *A. parasiticus*; yeasts; *Vibrio parahaemolyticus* (Bullerman et al., 1977; Conner and Beuchat, 1984; Beuchat, 1976). Statistical analysis also indicated spores of 33A were more susceptible to effects of the oils than 40B and 1623E ($p < 0.001$). A similar effect for the oils of garlic and onion has been reported previously (DeWit et al., 1979).

The effect of the oils on germination of *C. botulinum* strains was different from their effect on growth. Onion, garlic, origanum, and cinnamon were most effective inhibiting germination of 33A (Fig. 4). With all oils at ≥100 ppm germination was <4%. At 10 ppm, onion and garlic showed highest activity. These two oils produced 20% germination compared to > 50% germination with the rest of the oils. With ethanol germination was 83%.

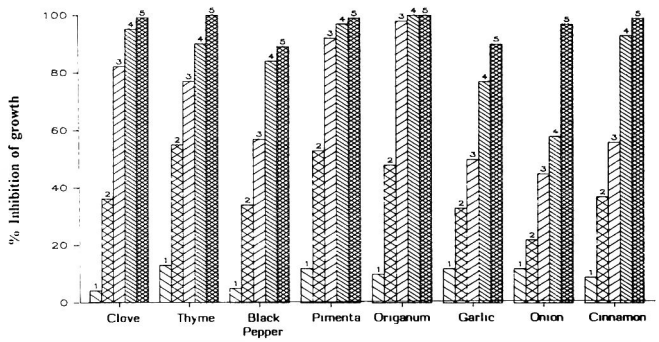

Fig. 3—Growth inhibition of C. botulinum *1623E by spice oils in Peptone Yeast Extract Agar media. Legend same as Fig. 1.*

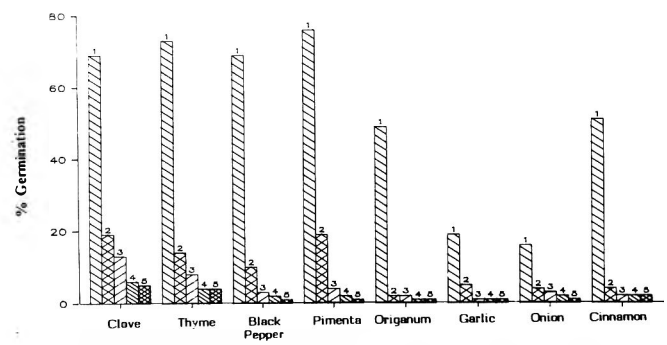

Fig. 4—Germination of C. botulinum *33A in the presence of spice oils. Legend same as Fig. 1.*

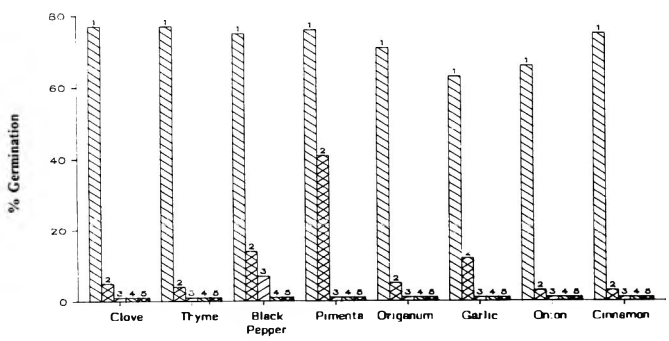

Fig. 5—Germination of C. botulinum *40B in the presence of spice oils. Legend same as Fig. 1.*

The effect of oils on germination of *C. botulinum* 40B is

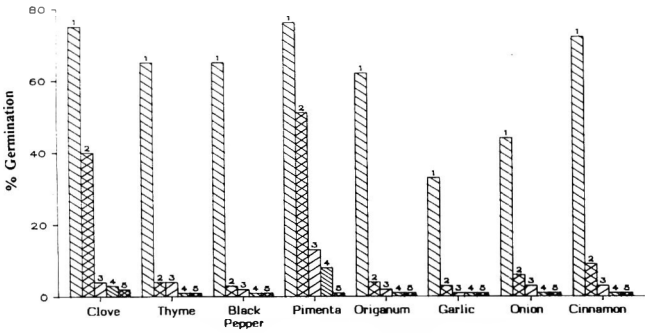

Fig. 6—Germination of C. botulinum *1623E in the presence of spice oils. Legend same as Fig. 1.*

depicted in Fig. 5. At concentrations of ≥ 100 ppm all oils were extremely effective in inhibiting germination of *C. botulinum* 40B. At 50 ppm, all ecxept pimenta oil allowed less than 15%. With pimenta oil germination was about 45%. At 10 ppm as with the previous strain, the oils of garlic and onion showed a little higher activity than the other oils.

The oils of pimenta and clove had the greatest effect on germination inhibition of strain 1623 E. At a concentration of 50 ppm, of these oils germination was 40–60% whereas with other spice oils tested at this concentration germination was 10% (Fig 6). At 10 ppm, the oils of garlic and onion were highly effective and germination was less than 50% compared to other oils which produced over 65% germination. At 100 ppm and above all the oils were highly effective with germination less than 4%.

Statistical analysis indicated that at ≥ 100 ppm all spice oils were highly inhibited germination of *C. botulinum* strains. At 50 ppm, however, oils of clove and pimenta had significantly lower activity than the other ($p \leq 0.05$). At 10 ppm, oils of garlic and onion had a greater effect on germination than the other ($p \leq 0.05$). Sulfur compounds in the oils of garlic and onion may be responsible for their high activity on germination. Our results suggest spice oils have an advantage over some other antibotulinal agents in preventing germination of *C. botulinum*. For instance, nitrite and sorbate do not have significant effect on germination of *C. botulinum* at the concentrations normally used in foods (Duncan and Foster, 1968; Smoot, 1981).

Comparing effects of the spice oils on germination to effects on growth, garlic and onion oils were extremely effective on germination, but, weakest on growth. This result may be caused by the difference in incubation times. The germination was monitored after 6 hr incubation whereas, growth was measured after 3 days incubation. During relatively long incubation some highly volatile antibotulinal components in these oils could evaporate from the media, leading to decrease in their concentration. As a result the spores germinate, outgrow, and grow with prolonged incubation. Other factors such as incubation temperature and media may have attributed to differences in effects of garlic and onion oils on germination as compared to growth.

CONCLUSION

THE HIGH EFFECT of spice oils on both growth and germination of *C. botulinum* spores at concentrations of 150–200 ppm indicate these oils could be used in foods in which *C. botulinum* is considered a health hazard. However, the sensory characteristics of such foods with the spice oils at the effective concentrations need to be studied before these spice oils can be recommended for use as antibotulinal agents.

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Lipase Mediated Synthesis of Low Molecular Weight Flavor Esters

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ABSTRACT

Screening 27 commercial lipases showed that enzymes from *Candida cylindracea, Pseudomonas fluorescens* and *Mucor miehei* (immobilized) promoted synthesis of selected low molecular weight esters in nonaqueous systems. Maximum production after 24 hr incubation was obtained with substrate concentrations of 0.05 mol/L for isopentyl acetate, 0.2 mol/L for ethyl butyrate and 0.3 mol/L for isopentyl butyrate. Yield of butyl butryate was almost 100% at acid substrate greater than 0.2 mol/L. Substrate inhibition was observed with *P. fluorescens* lipase but not with *C. cylindracea* or *M. miehei* lipases, up to 1 mol/L. Hexane, octane and decane could be used as reaction media except for ethyl butyrate synthesis where hexane was the medium of choice. Poor synthesis was achieved when methylene chloride was used.

INTRODUCTION

LOW MOLECULAR WEIGHT esters (LMWE) are used as flavors and fragrances in the food industry (Stofberg and Grundschober, 1987). For industrial use, they are usually produced by chemical synthesis and are not considered natural products. Thus their market value is less than esters from natural sources. Such esters, however, may be considered natural when produced by lipase-mediated syntheses.

Lipases have previously been screened for mediating synthesis of terpenoid and certain aliphatic esters (Kawamoto et al., 1987; Marlot et al., 1985; Langrand et al., 1988) but not for LMWE. LMW (water soluble; $<C_4$) substrates appear to have less affinity for these enzymes than longer chain length (water insoluble) substrates (Gatfield, 1986; Miller et al., 1988; Welsh and Williams, 1989a). This difference has been related to poor fit of substrate with the active enzyme site or to the poor solubility of LMW substrates in most nonaqueous reaction fluids commonly used in ester syntheses. Those studies, however, were conducted using a limited number of enzyme preparations. Enzyme source, substrate concentration and solvent type were shown to influence high molecular weight ester synthesis. The objectives of our study were to identify lipase sources that were suitable for LMWE synthesis and describe the effect of selected variables on the lipase-mediated syntheses of ethyl butyrate, butyl butyrate, isopentyl acetate and isopentyl butyrate.

MATERIALS & METHODS

LIPASE PREPARATIONS tested and sources were: *Rhizopus arrhizus, Candida cyclindracea*, and porcine pancreas from Sigma Chemical Co. (St. Louis, MO USA); *Aspergillus niger* AIE 60,000, *A. niger* ADF 75,000 and Lipase N from Amano Enzyme Co. (Troy, VA USA); immobilized *Mucor miehei* (IM 20) from Novo Industries (Wilton, CT USA); and Lipomod RD, Lipomod PC, Lipomod AC, *Candida lipolytica, Candida cylindracea, Pseudomonas fluorescens, Geotricum candidum, Rhizopus niveus, Rhizopus arrhizus, Rhizopus japonicus, Rhizopus javanicus, Rhizopus delemar, Aspergillus niger, Penicllium cyclopium, Penicillium roqueforti, Chromobacter viscosum*, porcine pancreas, *Humicola lanuginosa, Mucor javanicus*, and *Mucor miehei* from Biocatalysts Ltd (Pontypridd, Wales). All enzymes were used as received at 1% w/v for butyrate ester synthesis and 2% w/v for acetate ester synthesis.

Alcohol substrates used were: ethanol (95%, Commercial Alcohol Ltd., Montreal, Canada), butanol (Anachemica, Montreal, Canada) or isopentanol (Mallinckroft, Inc., Paris, KY USA). Acids used were glacial acetic acid (Anachemica, Montreal, Canada) or n-butyric acid (approximately 99%, Sigma Chemical Co., St. Louis, MO USA). Methylene chloride, hexane, octane (all from BDH Chemicals, Toronto, Canada) or decane (Aldrich Chemical Co., Milwaukee, WI USA) were used as reaction media.

Reaction medium (10 mL), enzyme and appropriate amounts of substrate were combined in serum vials (50 mL size) capped and incubated at 30°C with mixing (150 rpm). Hexane was the reaction medium except for the selection trials where methylene chloride, hexane, octane or decane were tested. Samples were taken after 0, 24 and 48 hr for most experiments or after 0, 1, 2, 3, 4, 5, 6 and 24 hr for experiments to determine initial reaction velocities (v_o). Samples were centrifuged (13,000 × *g* for 5 min at 24°C) to remove residual enzyme and stored at −10°C until analysis (usually within 24 hr). They were subsequently warmed to 24°C and analyzed according to previously described gas chromatography (GC) methods (Welsh and Williams, 1989a).

Screening trials to determine efficacy of the test enzymes for butyric acid ester synthesis were conducted using alcohol substrate concentrations (mol/L) of either: butanol, 0.26; isopentanol, 0.22; or ethanol, 0.41. The butyric acid concentration was 0.26 mol/L (250 μL of each substrate in 10 mL reaction fluid). Substrate concentrations for isopentyl acetate synthesis were 0.13 mol/L acetic acid and 0.07 mol/L isopentanol (75 μL of each substrate in 10 mL reaction medium).

Effect of substrate concentration on ester synthesis was determined using alcohol concentrations (mol/L): Isopentanol = 0.045, 0.09, 0.218, 0.417, 0.593 or 0.765; butanol = 0.054, 0.107, 0.26, 0.496, 0.713 or 0.91; or ethanol = 0.084, 0.168, 0.408, 0.779, 1.117 or 1.428. The acid substrate concentration was increased as alcohol concentration was increased to maintain alcohol to acid molar concentration ratio of 0.81 for isopropyl butyrate, 1.0 for butyl butyrate synthesis, 1.55 for ethyl butyrate and 0.52 for isopentyl acetate ester synthesis. Acid concentrations were (mol/L): butyric acid = 0.054, 0.107, 0.259, 0.494, 0.709 or 0.906; or acetic acid = 0.086, 0.171, 0.416, 0.794, 1.12 or 1.456 (volume additions of 50, 100, 250, 500, 750 or 1000 μL of each substrate to 10 mL reaction fluid). Acid substrate concentration was used to indicate substrate concentration.

Reaction medium selection experiments used substrate concentrations of (mol/L): isopentanol, 0.417; butanol, 0.496; or ethanol, 0.779; and butyric acid, 0.494 (500 μL of each substrate in 10 mL reaction fluid), for butyric acid ester syntheses. Substrate concentrations for isopentyl acetate synthesis were (mol/L): isopentanol, 0.068; and acetic acid, 0.129 (75 μL of each substrate in 10 mL reaction medium).

RESULTS & DISCUSSION

Screening trials

Under the standardized conditions, different lipases synthesized test esters to varying extents (Table 1). Lipases from *C. cylindracea* (CCL), *Ps. fluorescens* (PFL) and *M. miehei* (immobilized) (IM 20) gave yields of >20% for all test esters, while eight lipases did not synthesize butyl butyrate (<20% yield) within 48 hr. Differences in ester yields may be due, in part, to composition and preparation methods of the different lipases and the impact of water content of the different preparations. No attempt was made to correct lipases for amount of protein (enzyme) in the preparation. As manufacturers use

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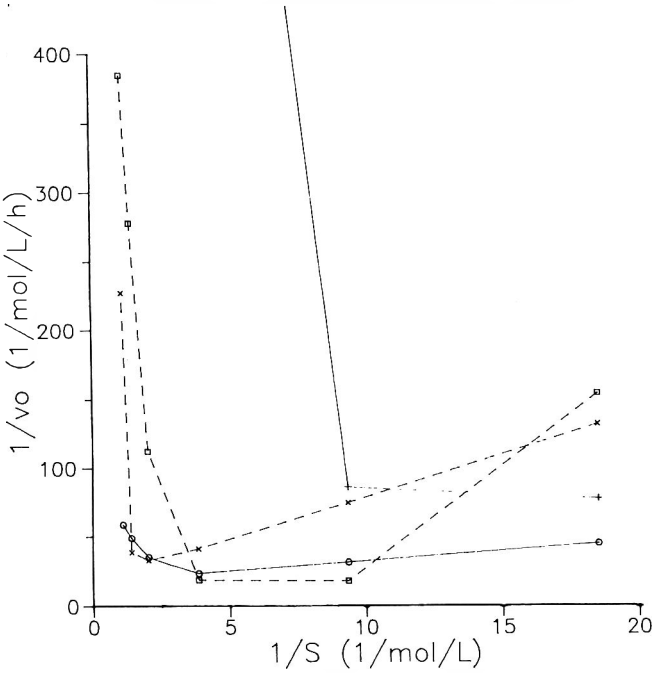

Fig. 1—Lineweaver-Burke plots of effect of substrate concentration on initial reaction velocity for synthesis of selected low molecular weight esters by Ps. fluorescens *lipase. (Legend: +----+ isopentyl acetate; ○——○ ethyl butyrate; ×----x isopentyl butyrate; □----□ butyl butyrate.)*

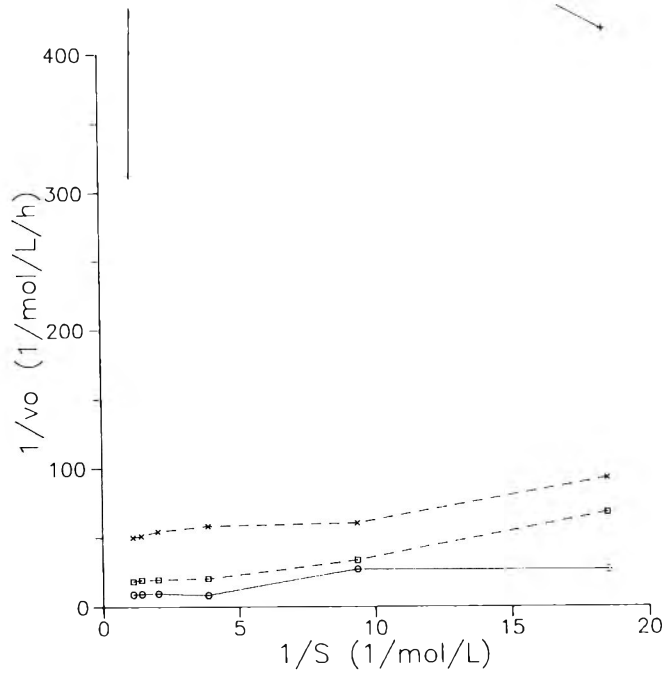

Fig. 2—Lineweaver-Burke plots of effect of substrate concentration on initial reaction velocity for synthesis of selected low molecular weight esters by M. miehei *lipase (immobilized). (Legend: +——+ isopentyl acetate; ○——○ ethyl butyrate; ×----× isopentyl butyrate; □----□ butyl butyrate.)*

Table 1—Effectiveness of lipase from different sources on synthesis of selected low molecular weight esters after 48 h incubation.

Enzyme source	Yield (%)[a] Ethyl butyrate	Isopentyl butyrate	Isopentyl acetate	Butyl butyrate
Lipase N[2]	28.6	TR[e]	2.7	2.0
A. niger	79.2	2.4	9.8	32.9
A. niger AIE 60,000	70.0	40.9	20.0	82.0
A. niger ADF 75,000	55.5	78.9	3.2	100.0
C. cylindracea[b]	79.2	86.5	75.8	96.7
C. cylindracea[c]	85.9	82.9	10.0	96.9
C. lipolytica	TR	TR	0.0	0.8
Chromobacter viscosum	84.8	81.4	0.0	100.0
G. candidum	TR	TR	0.0	1.5
H. lanuginosa	100.0	30.0	4.4	72.5
Lipomod AC	46.4	83.6	3.9	95.8
Lipomod PC	48.0	85.4	4.7	90.8
Lipomod RD	1.0	TR	0.0	1.1
M. javanicus	61.1	26.9	TR	94.5
M. miehei	48.6	89.0	13.6	95.1
M. miehei[d]	77.1	93.0	100.0	95.5
Pancreatic[b]	33.2	8.8	3.6	95.1
Porcine pancreas[c]	47.7	68.6	3.3	91.1
P. cyclopium	54.4	78.4	10.9	94.3
P. roqueforti	5.5	13.5	3.1	14.1
Ps. fluorescens	93.8	81.1	100.0	95.6
R. arrhizus[b]	TR	2.0	0.0	0.8
R. arrhizus[c]	0.0	17.6	0.0	61.9
R. delemar	81.5	2.0	0.0	5.5
R. japonicus	17.0	2.6	0.0	33.3
R. javanicus	85.7	TR	6.0	52.5
R. niveus	25.7	TR	0.0	1.2

[a] Yield = (mol ester produced/mol acid added) × 100; substrate concentrations are reported in the text.
[b] From: Biocatalysts, Ponypridd, Wales.
[c] From: Sigma Chemical Co. Ltd., St. Louis, MO USA
[d] From: Novo Industries, Wilton, CT USA (immobilized)
[e] TR = Trace amount detected

different preparation methods, the quantity of enzyme may vary from one lot to another thereby causing variability in overall activity. Similarly, differences in preparation methods could affect the amount of water in the reaction system. Ester syntheses are highly susceptible to water content of the reaction system (enzyme preparation and reaction fluid) (Yamane et al. 1989). Our studies were designed to examine the potential for a practical synthesis of these compounds and therefore no attempt was made to equate results with water or protein content. Any need to change the protein and water concentrations of preparations would impact the economics of practical synthesis. In addition, several of these preparations were crude preparations, and might contain contaminating esterases which could enhance relative activities.

Our results can be compared to the results of previous screening trials for the syntheses of other aliphatic and terpenoid esters. Lipases from *C. rugosa (C. cylindracea)* and *M. miehei* have been used to synthesize propionate and butyrate esters of isoamyl alcohol (isopentanol) and geraniol but only poorly synthesized acetate esters (Langrand et al., 1988). Gatfield, (1986) has shown that lipase from *M. miehei* could form propionate esters of alcohols up to C_{10} poorly while butyrate esters could be synthesized with alcohols greater than C_5. In addition, only 22 of 50 test hydrolases mediated the esterification of citronellol with 5-phenyl-valeric acid (Kawamoto et al., 1987), while eight lipases, immobilized on hydrophilic supports, mediated synthesis of geraniol butyrate (maximum yield = 74% by *C. cylindracea* lipase) and geraniol laurate (maximum yield = 64% by hog pancreas lipase). Yields varied with enzyme used (Marlot et al., 1985). A comparison of eight lipases showed enzyme from *C. cylindracea* was best suited for synthesis of carbohydrate esters (Seino et al., 1984).

The initial reaction velocity (v_o) data for PFL (Fig. 1) and IM 20 (Fig. 2) further demonstrated the variability in effectiveness of enzymes from different sources. PFL-mediated synthesis of butyrate esters was inhibited when acid substrate concentration exceeded about 0.3 mol/L for isopentyl butyrate, 0.2 mol/L for ethyl butyrate, and 0.25 mol/L for butyl butyrate syntheses. IM 20 was not inhibited by substrate concentrations up to 1 mol/L. This could be caused by the effect of immobilization on enzyme activity, as discussed below. Isopentyl acetate synthesis was inhibited at substrate concentrations greater

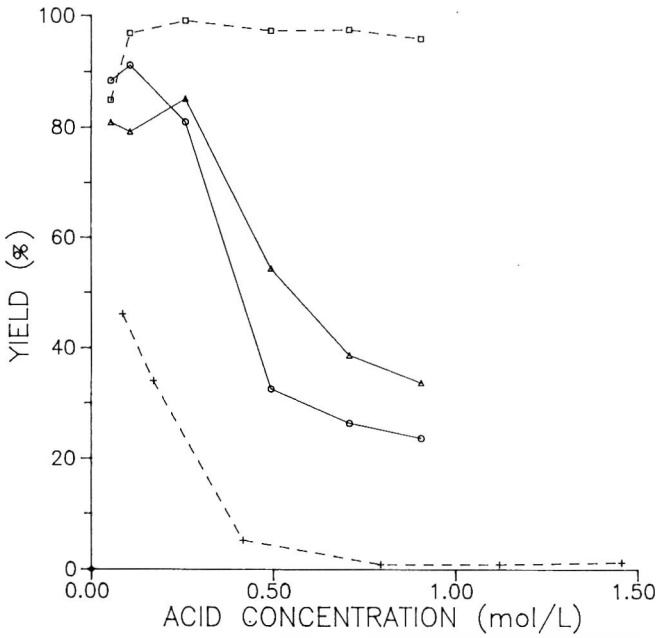

Fig. 3—Effect of substrate concentration on yield of selected low molecular weight esters by M. miehei *lipase (immobilized) after 24 hr incubation. (Legend: +----+ isopentyl acetate; ○———○ ethyl butyrate; △——△ isopentyl butyrate; □----□ butyl butyrate.)*

Table 2—V_{max} and apparent K_m for acid utilization during the production of four low molecular weight esters by three lipases using hexane as reaction medium

Enzyme	Constant	Ethyl butyrate	Butyl butyrate	Isopentyl acetate	Isopentyl butyrate
Mucor miehei	V_{max}[a]	0.109	0.051	0.002	0.019
(immobilized)	K_m[b]	0.140	0.092	0.390	0.042
Candida	V_{max}	0.145	0.120	0.006	0.080
cylindracea[c]	K_m	0.123	0.200	0.145	0.160
Pseudomonas	V_{max}	0.043	0.056	0.014	0.030
fluorescens	K_m	0.055	0.075	0.258	0.140

[a] V_{max} = moles/L/hr.
[b] K_m = mol/L.
[c] From Biocatalysts Limited, Pontypridd, Wales.

Table 3—Yields of selected low molecular weight esters produced using C. cylindracea *lipase with methylene chloride, hexane, octane or decane as reaction medium after 24 h incubation*

	Yield (%)[a]			
Ester	Methylene chloride	Hexane	Octane	Decane
Isopentyl acetate	0.0	61.8	58.4	34.8
Isopentyl butyrate	41.0	96.6	97.9	96.7
Ethyl butyrate	5.7	97.6	51.0	0.0
Butyl butyrate	0.5	100.0	100.0	100.0

[a] Yield = (mol ester produced/mol acid added) × 100; Substrate concentrations are reported in the text.

than 0.05 mol/L, in both cases. For all test syntheses, CCL results for v_o were similar to those for IM 20 (Data not shown). This similarity was also noted by Deleuze et al. (1987). Data for V_{max} and apparent K_m (calculated using one half V_{max}) (Table 2) for the four syntheses by CCL, PFL and IM 20 further emphasize the need to select the correct lipase for the synthesis required.

The effect of immobilization on the enzyme's synthetic capacity is shown in Table 1. IM 20 synthesized ethyl butyrate (yield = 77%) and isopentyl acetate (yield = 100%), however, nonimmobilized *M. miehei* lipase produced ethyl butyrate at 48.6% yield and isopentyl acetate 13.6%. In contrast, other researchers have shown IM 20 converted less isopentanol and geraniol to acetate and propionate esters than non-immobilized *M. miehei* lipase (Langrand et al., 1988). Furthermore, comparisons of data obtained with several enzymes immobilized on hydrophilic supports (Marlot et al., 1985) with their non-immobilized counterparts (Langrand et al., 1988) showed that immobilization reduced enzyme activity. These effects may have been partly due to differences in enzyme type and/or lot, but it appears enzyme activity was altered by immobilization. These changes could enhance or decrease activity depending on changes in configuration or substrate dissociation to the active site. Implications of enzyme immobilization have been reported (Lavayre and Baratti, 1982; Linko and Linko, 1985).

Lipase preparations from different sources/suppliers can have widely differing activities as seen by comparing results for two CCL's, three *A. niger* lipases, two *R. arrhizus* or two pancreatic lipases (Table 1). Differences were observed for two *M. miehei* and two porcine pancreas lipases for synthesis of isopentyl and geranyl esters of acetic, propionic and butyric acids and for transesterification reactions (Langrand et al., 1988). These differences may be due, in part, to differences in microbial strain, processing methods or extenders used.

Substrate concentration effects

Substrate parameters such as concentration, size, and/or presence of side chain groups have been shown to affect large molecular weight ester synthesis. The effect of substrate concentration (indicated by acid concentration) on LMWE synthesis can be observed in yield data, after 24 hr for IM 20 (Fig. 3). Ethyl butyrate and isopentyl butyrate syntheses maintained high yields (>80%) up to substrate concentration 0.3 mol/L then yield decreased as substrate concentration increased. On the other hand, isopentyl acetate synthesis had low yield (45%) at 0.05 mol/L substrate and yield decreased as substrate concentration increased. Similar results were obtained for syntheses mediated by PFL and CCL.

The effect of acid substrate molecular size on ester synthesis can be seen by comparing isopentyl acetate and isopentyl butyrate data for the three enzyme sources examined: isopentyl acetate synthesis had lower yields and v_o than isopentyl butyrate synthesis (Fig. 1–3). These results agree with previously reported results where acetic acid and propionic acid were shown to prevent ester syntheses (Gatfield, 1986; Welsh and Williams, 1989a), while butyric acid was shown to be inhibitory to ethyl butyrate synthesis (Gilles et al., 1986). Similarly, 2-(p-chlorophenoxy)-propionic acid was inhibitory to its butyl ester synthesis (Cambou and Klibanov, 1984).

The molecular size of the alcohol substrate was also shown to affect these syntheses. Less ethyl butyrate was produced at high alcohol and acid concentrations than butyl butyrate, but initial reaction rates were similar (Fig. 1–3). Ethanol has been shown to affect ethyl butyrate syntheses using *A. niger* and porcine pancreatic lipases (Welsh and Williams, 1989b) and *M. miehei* lipase (Gatfield, 1986, Miller et al., 1988). A polyethylene glycol-modified lipoprotein lipase from *Ps. fluorescens* was also inhibited by water-miscible alcohol substrates but activity steadily increased when concentration of water-immiscible alcohol substrates increased (Takahashi et al., 1984).

The effect of side chains of the alcohol substrate on LMWE synthesis can also be seen from these data (Fig. 3). Isopentyl (3-methyl-1-butanol) butyrate synthesis had overall lower yields and reduced yield at higher substrate concentration than butyl (1-butanol) butyrate synthesis. These results were paralleled by the v_o data (Fig. 1 and 2). The addition of side groups to either the acid or alcohol substrate has been shown to reduce enzyme activity (Gilles et al., 1987; Miller et al., 1988; Eigtved et al., 1986, 1987). Generally, this effect was eliminated when the side group was located further from the α-carbon.

Solvent selection effects

The effect of solvent selection on yield for LMWE syntheses by CCL is shown in Table 3. For hexane, octane and decane,

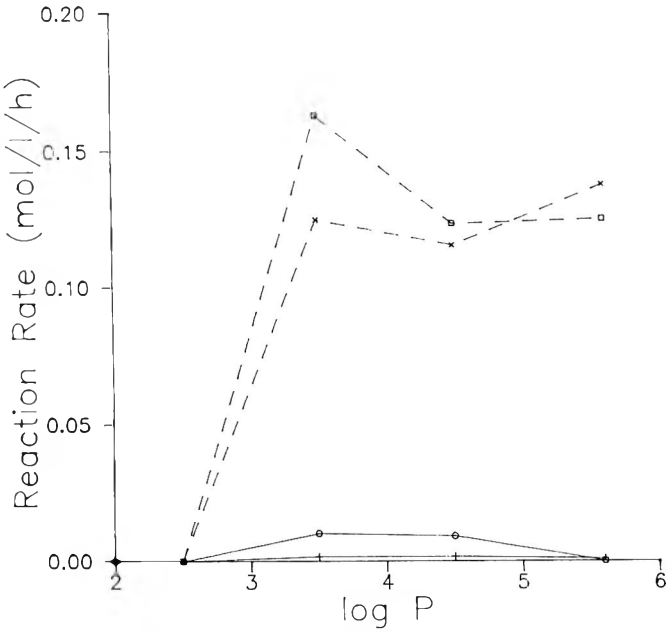

Fig. 4—Initial reaction rate for selected low molecular weight ester syntheses by C. cylindracea *in methylene chloride (log P=2.5), hexane (log P=3.5), octane (log P=4.5) or decane (log P=5.5). (Legend: +———+ isopentyl acetate; ○———○ ethyl butyrate; ×----× isopentyl butyrate; □----□ butyl butyrate.)*

isopentyl butyrate and butyl butyrate syntheses had yields of almost 100% while isopentyl acetate and ethyl butyrate yields decreased as chain length of the reaction medium increased. Syntheses conducted in methylene chloride produced less ester than similar reactions in the other test solvents. In addition, CCL-mediated synthesis of all test esters had slower v_o in methylene chloride than in the other reaction media (Fig. 4). Similar results were obtained with IM 20 and PFL.

The solvent dependency of butyl butyrate and isopentyl butyrate syntheses was similar to that described for other hydrolase-mediated esterification reactions. Water-miscible and partially water immiscible solvents which disperse small amounts of water such as methylene chloride (log P=2.5; where log P=log of the extraction coefficient for the test compound between water and octanol) were shown to impede these syntheses while water-immiscible solvents such as hexane, octane and decane (log P=3.5, 4.5 and 5.5 respectively) enhanced them (Zaks and Klibanov, 1985; Koshiro et al., 1985; Takahashi et al., 1984). This difference was postulated to result partly from stripping of essential water from the enzyme by the more hydrophilic solvents and partly from the solvent directly affecting the enzymatic process (Zaks and Klibanov, 1988). Other studies have identified physicochemical parameters (Reslow et al., 1987) and a set of guidelines (Laane et al., 1987) for solvent selection.

The data for syntheses with C_2 substrates (Table 3, Fig. 4) deviate from expected results using the proposed rules of Laane et al. (1987). An experiment was conducted to determine if this difference was due to substrate inhibition or failure of the substrate to reach the active site of the enzyme. Serum vials were prepared containing 0.1g CCL, 10 mL hexane, octane or decane, and substrate. Substrate consisted of 500 μL butyric acid, and either 500 μL ethanol, 500 μL butanol or 250 μL each of ethanol and butanol. The samples were incubated 24 h then sampled and analyzed as previously described. Syntheses with ethanol had yields of 70% in hexane, 10.9% in octane, and 5.2% in decane while syntheses with butanol had yields of 94.5% in hexane, 100% in octane and 99.6% in decane. On the other hand, ethyl butyrate and butyl butyrate were both formed in almost equal amounts for the reaction with the mixed alcohol substrate in all test reaction media (total yields of both esters were: 97.1% in hexane, 91.2% in octane and 93.6% in decane). It appears that C_8 and C_{10} reaction media prevented ethanol from entering the enzyme active site, unless accompanied by a co-solvent (butanol) at our low mixing rates (150 rpm). Further research is needed to elucidate a more complete set of guidelines for solvent selection for the lipase-mediated synthesis of LMWE.

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Prediction of Flavor Retention during Spray Drying: An Empirical Approach

W.E. BANGS and G.A. REINECCIUS

ABSTRACT

A rapid, semi-quantitative prediction model was developed for retention of volatile organic flavoring compounds during spray drying. Nonequilibrium vapor phase flux and effective (liquid phase) diffusion coefficients of lemon oil and/or butanol isomers (n-, sec-, tert-) correlated with spray dryer retention. Prediction of retention of these compounds was dependent on the specific sample matrix and dryer.

INTRODUCTION

SPRAY-DRIED FLAVORINGS are economically important to the food supply (Brenner, 1983). Unfortunately, production of these materials is largely an empirical science due to the complexities of the volatile retention and water-removal processes. No research has explicitly defined drying characteristics of the complex polymers which exist as wall materials. Retention of volatile organic compounds during evaporative removal of water, (the carrier solvent), via spray drying was expected to be even more difficult to define.

Various researchers (Zakarian and King, 1982; Kerkhof and Thijssen, 1975; Kerkhof, 1974; Kerkhof and Thijssen, 1977). have examined computer simulation models in an attempt to predict retention of volatile organics during spray drying. While no universally accepted models have evolved which would allow for absolute, a priori predictions of volatile retention in spray drying, a solid foundation has been established. Applications of the concepts are now clearly feasible. The purpose of our study was to apply these concepts for a rapid, empirical approach to the complex phenomenon of aroma retention during the spray drying process.

MATERIALS & METHODS

Wall material selection

Three polysaccharides were chosen as standard wall materials for study: Capsul (National Starch and Chemical Corp., Bridgewater, NJ); gum acacia (Fritzsche Dodge and Olcott, Inc., NY, NY); and a maltodextrin with a dextrose equivalent of 10 (M-100; Grain Processing Corp., Muscatine, IA).

Core material selection

Three considerations were used for selection of a volatile core material: it should be readily available, derived from a natural flavoring source and indicative of a "real" system (i.e., historical usage as a core material). For these reasons lemon oil obtained by cold pressing lemon peel was selected (Universal Flavors, Indianapolis, IN). When emulsion instability problems resulted using lemon oil, aqueous soluble concentrations of butanol isomers (n-, sec-, tert-; Sigma Chemical Co., St. Louis, MO) were used.

Water removal processes

In order to screen small quantities (< 30g) of various combinations for their retention of volatile core material following spray drying, a bench-top spray dryer was constructed. A six liter Erlenmeyer flask was inverted and tapped into the base for nozzle placement. Ground glass joints were used to physically attach the two-fluid atomizer and a Buchi cyclone was used for powder collection. Concurrent, tangential heated air was provided by a variable temperature hot air gun specified to deliver 9 SCFM of air at 400°C (Cole-Parmer, Chicago IL).

A bi-lobe positive displacement micro-pump (Cole Parmer, Chicago, IL) was used to deliver aqueous feed material. Constant air temperatures were maintained for all drying trials using 220 ± 5°C inlet and 100 ± 5°C outlet. These conditions allowed an evaporation rate of about 750 g water/hr. Ambient dryer feedstock temperatures (ca. 26°C) were maintained for all drying trials.

A Niro Utility Model spray dryer (Niro Atomizer Ltd., Columbia, MD), using a centrifugal atomizer, was used for comparison. Inlet and exit air temperatures were the same as for the mini-dryer. Under these conditions about 11.5 kg water/hr was removed. Ambient temperature feedstocks were fed into the dryer using a 1 L separatory funnel (headpressure feed).

Dryer sample preparation

Four to six hours prior to drying, the wall material was hydrated in distilled water at concentrations of 33% and 67% (w/v-gum arabic was studied only at 33% solids). Twenty percent (solids basis) lemon oil or 0.33% (water basis) butanol(s) were incorporated following the hydration procedure and emulsion formation was achieved by the use of a magnetic stir plate and/or a Greerco laboratory mixer (Greerco Corp., Hudson, NY).

Analysis and sample preparation

A static headspace (using a heated six-port valve and a 2 mL sampling loop)/gas chromatographic (GC) technique was developed to measure gas phase flux of volatile organic compounds (Bangs, 1985). Gum arabic, Capsul and M-100 emulsions were analyzed using solids levels equal to those used for drying. Lemon oil or butanol (n-, sec-, tert-) concentrations indicative of dryer feed concentrations were used (20%, w/w dry solids basis and 0.33% w/v water basis, respectively). Ten mL of emulsion was pipetted into an 11-dram vial, capped and examined for volatile headspace content at various sampling times (0–2.0 hr). Each analysis was run in triplicate and reported values are average percentages of an external standard (%ESTD) computed as follows:

$$\%\text{ESTD} = \frac{\text{Sample avg vapor phase content}}{\text{Water avg vapor phase content}} \times 100$$

The external standard was a vortex-mixed sample of lemon oil or butanol in water equal to that in the samples containing wall materials. The external standard data were analyzed in duplicate for each sample.

The GC-acetone precipitation method of Subramaniam (1984) was used to analyze dried samples for volatile retention. Dryer feed samples as well as rehydrated dried samples were analyzed for lemon oil or butanol, using 2-nonanone or n-pentanol, respectively, as internal standard. Percent retention was recorded as the ratio of final to initial core material.

A Hewlett-Packard Model 5880 gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a single FID was used for quantitative analysis. A 25 m × 0.25 mm OV-17 bonded phase fused silica capillary column (Hewlett Packard, Avondale, PA) was operated isothermally (100°C) using hydrogen as carrier (40 cm/sec linear velocity) to quantify lemon oil. All injections were in split mode (60 mL/min). A 30 m × 0.32 mm DB-1 bonded phase column (J & W Scientific, Rancho Cordoba, CA) was operated isothermally (40°C) with hydrogen as carrier (30 cm/sec linear velocity) to quantify butanols.

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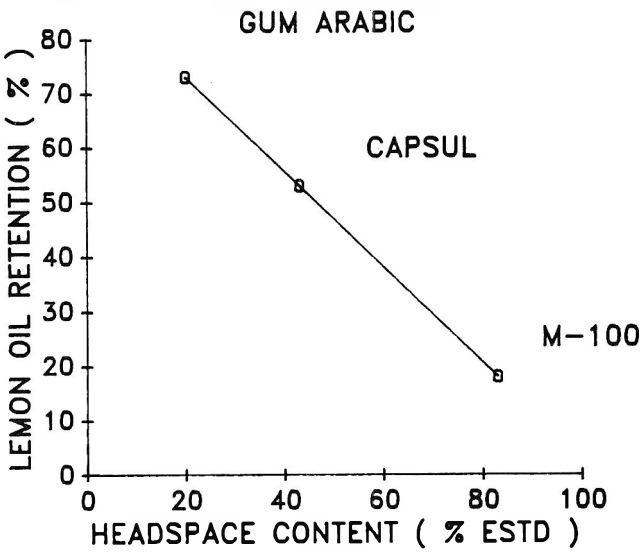

Fig. 1—Relationship between headspace content of lemon oil and its retention during spray drying using a minidryer.

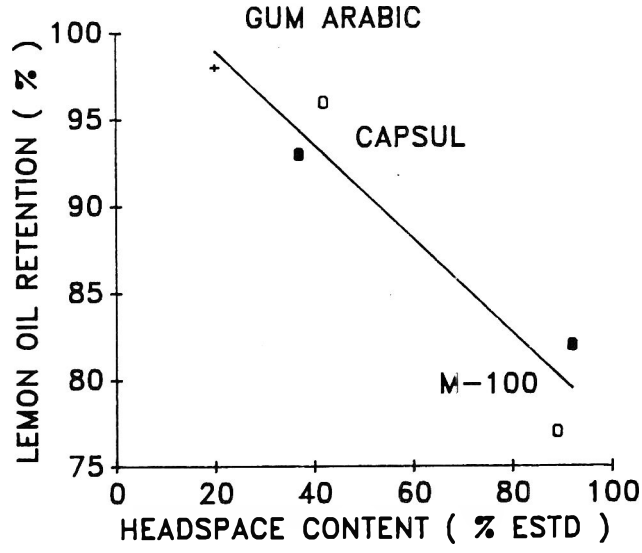

Fig. 2—Relationship between headspace content of lemon oil and its retention during spray drying using a Niro dryer (open symbols 33% infeed solids; solid symbols 67% infeed solids).

Table 1—Lemon oil headspace analysis and mini-dryer retention data

Sample (33% w/v Carrier Solids)	Equilibrium (hr)	%EStD[a]	Mini drier retention % A/A_0[b] Trial 1	Trial 2	Avg
Gum Acacia	0	22.0	72.9	70.2	71.5
	0.5	89.3			
	1.0	90.1			
	1.5	91.9			
	2.0	95.9			
M-100	0	85.3	23.3	12.9	18.1
	0.5	94.0			
	1.0[c]	99.0			
Capsul	0	47.2	52.2	53.7	53.0
	0.5	87.7			
	1.0	89.7			
	1.5	90.0			

[a] Percent lemon oil present in headspace sample relative to equilibrium value (% ESTD)
[b] Amount of lemon oil remaining following drying/initial amount
[c] Emulsion broke

Table 2—Physical properties and encapsulation agent retention of butanol using the Niro dryer

Sample (33% w/v)	Compound	% EStD at zero time	Diff. coefficient Dj, eff × 10^6 (cm^2/sec)	Dryer retention % A/A_0[1]
Gum Acacia	n-butanol	11.5	2.93	32.6
	sec-butanol	13.9	3.57	19.2
	tert-butanol	14.3	3.01	46.1
Capsul	n-butanol	12.8	3.80	27.7
	sec-butanol	14.4	3.86	13.8
	tert-butanol	14.1	3.54	40.0
M-100	n-butanol	15.5	4.56	20.6
	sec-butanol	18.6	4.37	5.5
	tert-butanol	20.0	3.31	14.0
Water	n-butanol	29.7[b]	5.54	0
	sec-butanol	33.4[b]	5.53	0
	tert-butanol	33.5[b]	5.38	0

[a] Amount of butanol remaining following drying/initial amount
[b] No sample mixing

Diffusivity

An effective diffusivity term associated with flux of volatile core material from an emulsion sample was determined via the diaphram cell technique (Lightfoot and Cussler, 1965). A 0.20 μm membrane filter (Nucleopore Corp., Pleasanton, CA) was used to isolate the two cell chambers and a binary system of known diffusivity (1% methanol in water) was used for calibration.

Due to lemon oil emulsion instability, butanol isomers (n-, sec-, tert-) were used as model diffusants. Combinations representative of those in the drying experiments were used to determine isothermal (26°C; dryer infeed temperature) effective diffusivity values.

RESULTS & DISCUSSION

THE HEADSPACE lemon oil in 33% (w/v) gum acacia, Capsul and the DE 10 (M-100) maltodextrin quiescent solutions were determined as a function of time (Table 1). Equilibrium vapor phase concentrations of lemon oil volatiles were attained in about 30 min at ambient temperature (ca.26°C).

Sample emulsions were prepared exactly as for the headspace analyses and dried in duplicate using the mini-dryer. The lemon oil retention data from these trials are also shown in Table 1.

A plot of retention versus headspace content of lemon oil using the time "O" data for the systems studied is shown in Fig. 1. An excellent correlation coefficient (R^2) of 0.99 was obtained with the linear regression line very nearly passing through ordinate and abscissa points of 0, 100, and 100, 0. Thus, to predict retention of lemon oil during drying, simply conduct a headspace analysis (reported as % EStD) and subtract that value from 100%. Apparently the critical concept was use of nonequilibrium headspace data, not the equilibrium value reported by Voilley et al. (1977).

Results of the scale-up study using the Niro dryer is shown in Fig. 2. A correlation R^2 of 0.93 was found for the same parameter although there was a slight shift in the regression line slope from the "ideal" obtained with the mini-dryer. The reasons for differences might be the amount of occluded air and particle size were different between the two. This could alter dryer retention due to differences in effective diffusion paths. In addition, air flow patterns for the two dryers were quite different and might have contributed to the substantial differences in volatile retention.

This empirical approach to retention predictions of volatiles using nonequilibrium headspace data is fairly similar to the efforts of Thijssen and Rulkens (1968), Kerkhof (1974, 1977) or Kerkhof and Thijssen (1977). Assuming most volatiles are lost from the atomized droplet during a constant rate drying period, as Zakarian and King (1982) hypothesized, determination of isothermal effective diffusivity values from a stagnant aqueous matrix at the dryer feed temperature should provide qualitative predictions. This assumes all other parameters are held constant and the selective diffusion retention theory Thijssen (1965) governs volatile loss.

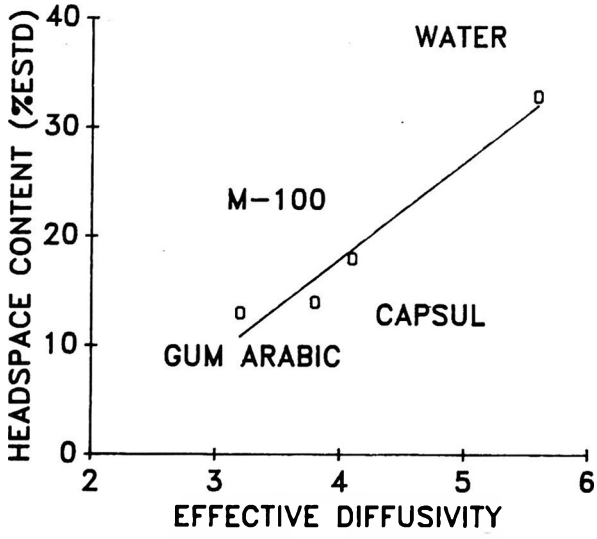

Fig. 3—Relationship between effective diffusivity (cm²/sec X 10⁶) of butanols and observed vapor phase headspace flux (headspace content).

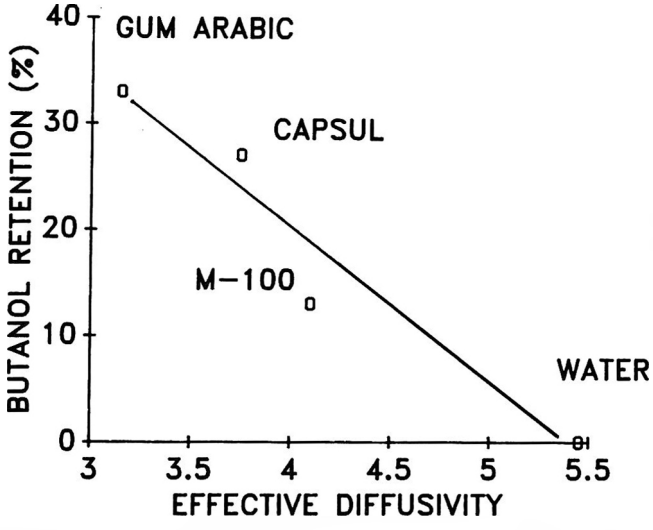

Fig. 4—Relationship between effective diffusivity (cm²/sec X 10⁶) of butanols and their retention after drying in the Niro dryer.

The non-equilibrium headspace lemon oil content may have fortuitously correlated with dryer retentions. Or it may have provided diffusivity information which allowed predictive determinations. To determine this, simple isothermal, binary, effective diffusivity data of butanol isomers were collected using the diaphragm cell technique.

In addition, aqueous samples incorporating the three standard wall materials and butanol isomers were prepared and dried in the Niro dryer. Retention, headspace contents and effective diffusivity are show in Table 2. An excellent correlation coefficient was derived for linear regression of effective diffusivity on headspace content ($R^2 = 0.96$) as shown in Fig. 3 and for effective diffusivity and drier retention ($R^2 = 0.94$) as shown in Fig. 4. A poorer correlation ($R^2 = 0.88$) was found between headspace and drier retention in the Niro drier (Fig. 5). Note that contrary to the work of Rulkens and Thijssen (1972), equal retentions of the three butanol isomers was not achieved. Secondary butanol resulted in the poorest retention following spray drying in all cases. However, with $R^2 = 0.88$, this would still be fairly good for choosing wall materials.

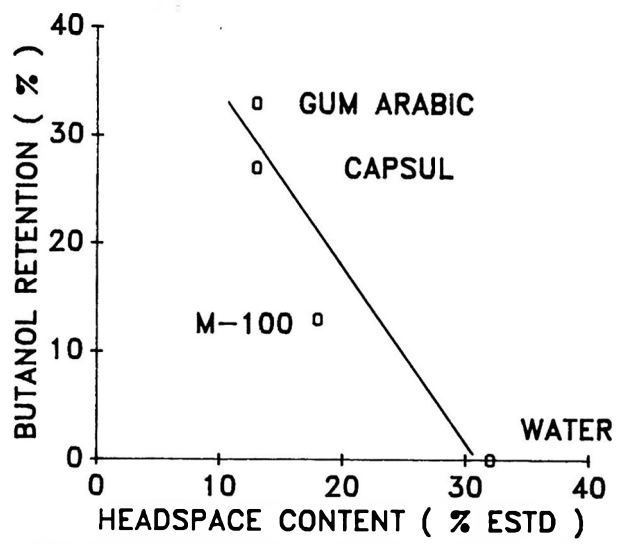

Fig. 5—Relationship between average headspace contents of butanols and their retention after drying in the Niro dryer.

CONCLUSIONS

THE IMPORTANCE of losses of volatiles associated with their diffusive flux in the atomization zone of the spray dryer was reaffirmed. At the infeed temperature examined, the primary variable affecting retention during drying appeared to be the effective diffusivity of these flavoring agents from the expanding liquid film and stagnant droplets. Once a selectively permeable membrane was formed at the droplet surface, the diffusion of volatiles into the drying medium was greatly hindered. In order to facilitate selection of wall materials ("carrier") for manufacturing spray-dried flavorings, a simple, rapid measurement of headspace volatiles was developed.

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Interaction of β-cyclodextrin with Enantiomers of Limonene and Carvone

YUEH-ING CHANG and GARY A. REINECCIUS

ABSTRACT

The inclusion complexes of β-cyclodextrin with two pairs of chiral flavor compounds, limonene and carvone, were used to evaluate the chiral interaction. Thermal transition was examined by DSC and crystalline properties by x-ray diffractometry. The overall DSC thermograms and X-ray diffraction patterns were very similar between the chiral isomers. A difference of about 3 joules/g complex was observed between the chiral isomers of both limonene and carvone. The only apparent difference in X-ray diffraction patterns between chiral complexes occurred at a Θ of 11.8°.

INTRODUCTION

FOOD is a complex mixture of proteins, carbohydrates, lipids, water and other organic and inorganic compounds which can interact with and bind flavor compounds. This binding by specific constituents leads to loss of available flavor and change in sensory qualities. Various studies have been conducted to examine this in model systems O'Neill and Kinsella, 1987; Mills and Solms, 1984; King, 1978; Saleeb and Pickup, 1978; Wyler and Solms, 1981. Binding of flavor compounds in foods is governed by the qualitative and quantitative nature of flavor compounds and food constituents. Consequently, it is extremely complicated to predict binding interactions.

A property which has received very limited attention in research reports, and which may play a role in interaction is the chiral property of flavor compounds. Since certain food components, e.g., many amino acids, proteins, carbohydrates and lipids, are chiral in nature, they will provide an asymmetric environment for flavor compounds. The degree of interaction with the chiral constituents in foods may be different between enantiomeric flavor compounds. The objective of our study was to assess chiral interactions by examining a model system (using differential scanning calorimetry, DSC, and X-ray diffractometry XRD) comprised of β-cyclodextrin and various chiral flavor components.

MATERIALS & METHODS

Chemicals

β-cyclodextrin was obtained from Fritzsche Dodge & Olcott (New York, NY) as a gift. The four chiral compounds, R(+)-limonene (97%), S(−)-limonene (92%), R(+)-carvone (96%) and S(−)-carvone (98%) were obtained from Aldrich Chemical Company (Milwaukee, WI).

Inclusion complex formation

β-cyclodextrin (4g) was dissolved in 90 mL boiling distilled water to form a clear solution, and then cooled to room temperature. The test chiral compound (about 3.5 x 10^{-3} mole) was well dispersed in 10 mL distilled water in a minihomogenizer before adding it to β-cyclodextrin solution. The mixture was then vigorously stirred overnight on a magnetic stirrer at room temperature. The inclusion complex was filtered through Whatman #4 filter paper on a Buchner funnel. The precipitate was air dried two hours, frozen in a dry ice freezer (−76°C), and dried overnight under vacuum ca. 10 μm Hg

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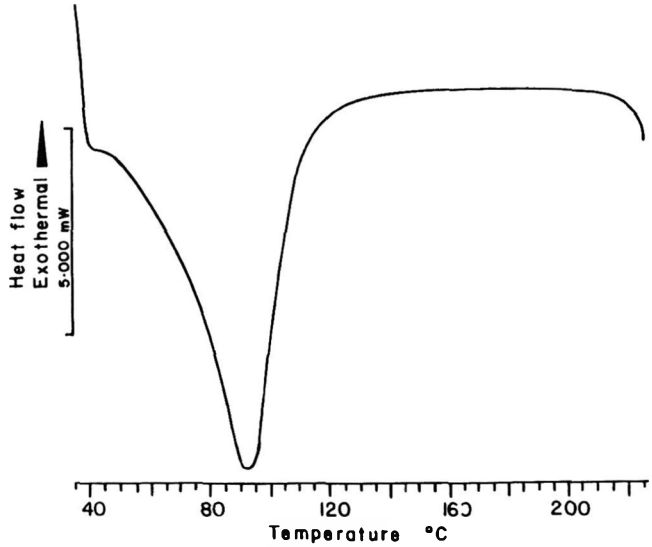


Fig. 1—DSC thermogram of pure β-cyclodextrin.

in a Virtis freeze drier (Virtis Research Equipment Company, Gardiner, NY). Dried samples were stored in a desiccator until analysis. Samples were analyzed for thermal transition properties by DSC, and for crystalline properties by XRD.

Differential scanning calorimetry

The inclusion complex sample (ca. 8 mg) was weighed into a tared aluminum pan. The sample was sealed in the pan with a lid crimped into position. The lid was pre-pierced for venting moisture or other volatiles to prevent deformation of the pan. A Mettler Model TA 3000 Differential Scanning Calorimeter in the Department of Chemical Engineering and Material Sciences, University of Minnesota (Minneapolis, MN) was used for thermal transition analysis.

The encapsulated sample was scanned from 35 to 200°C at 10°C/min with dried nitrogen purge to prevent possible thermally-induced oxidation. Heat capacity was measured vs temperature. Calibration of the instrument was by use of an inert Indium standard to standardize the temperature scale and specific heat capacity. The enthalpy was calculated by the integration function of the data processor.

X-Ray diffractometry

Sample (ca. 1 g) was placed in an aluminum holder and the sample surface was leveled with a clean smooth glass plate. XRD pattern of inclusion complex was recorded on a Siemens Model D 500 Diffractometer in the Department of Chemical Engineering and Material Sciences, University of Minnesota (Minneapolis, MN). CuK_α radiation was used as the energy source, at 40 KV and 30 mA. Two Θ/Θ symmetry mode was employed for scanning, and 2 Θ was scanned from 5 to 30° at 2° per minute. Slit widths were 1°, 1°, 1°, 0.15° and 0.15°, respectively. Time constant was 1 sec and sensitivity was set at 4 x 10^3 cps.

RESULTS & DISCUSSION

WHEN TWO interacting components are chiral in nature, the difference in chiral property of one may affect the interaction

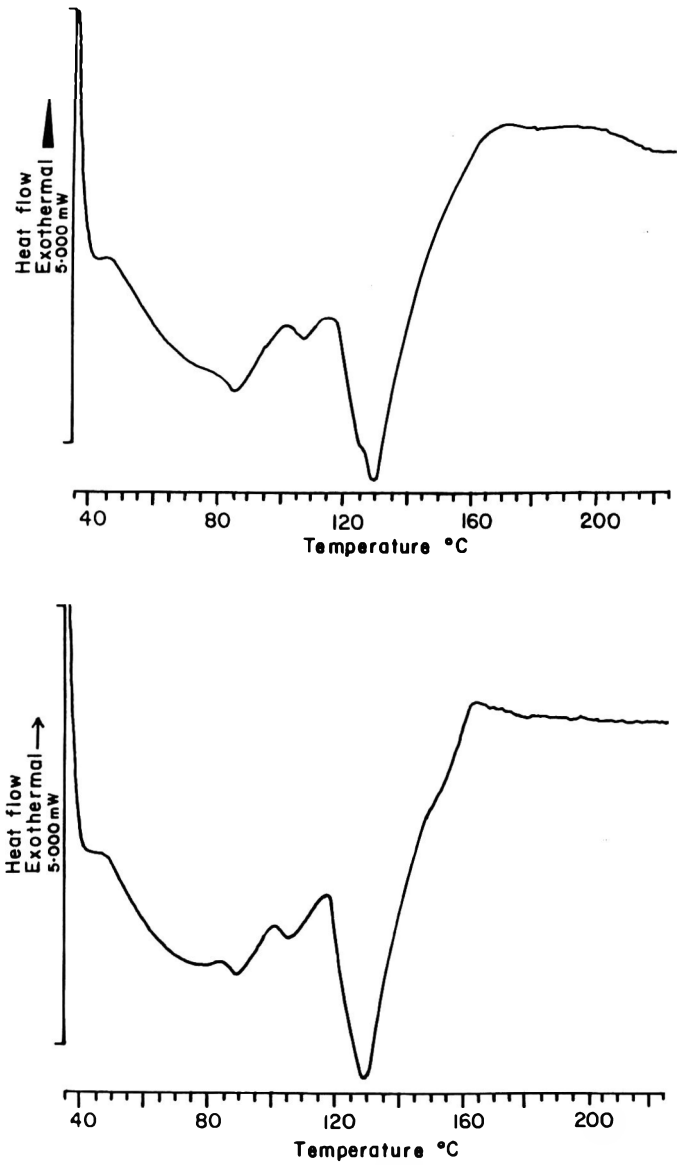


Fig. 2—DSC thermograms of chiral limonene/β-cyclodextrin complexes. [Top—R(+), Bottom—S(−)]

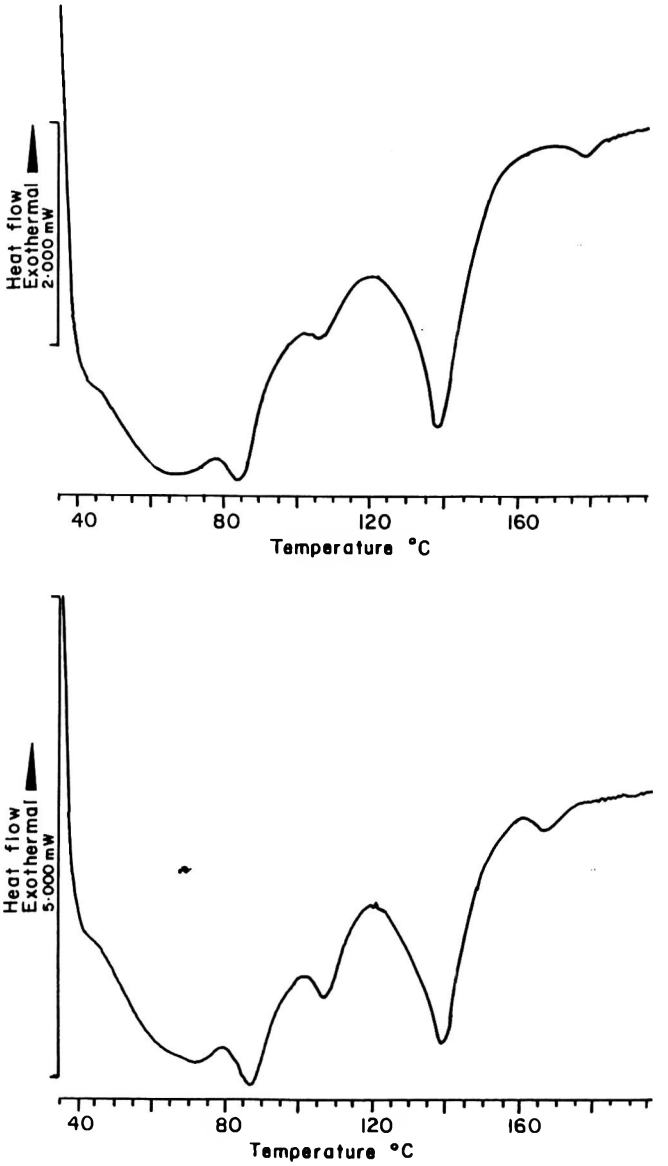


Fig. 3—DSC thermograms of chiral carvone/β-cyclodextrin complexes. [Top—R(+), Bottom—S(−)]

rate or stability of the interaction complex formed. In our study, β-cyclodextrin, a chiral cyclic oligosaccharide, was used to form inclusion complexes with chiral limonene and carvone. Inclusion complexes were formed by the solution method and all four complexes formed (R(+)−limonene, S(−)−limonene, R(+)−carvone and S(−)−carvone) had similar yields--3.90 g (ca. 87%).

The thermogram of pure β-cyclodextrin is presented in Fig. 1. Only one peak at 93°C was observed, indicating heat absorption began at 40°C and terminated at 135°C, probably caused by vaporization of moisture. The thermal transition profiles of R(+)− and S(−)−limonene inclusion complexes are shown in Fig. 2. The overall profiles were similar with the peak temperature at 130°C. Since the temperature and heat exchanged during a change of state is a thermodynamic constant, we speculate that maximal dissociation of the inclusion complex took place at this temperature and, thus, maximal heat was absorbed. The enthalpies of this peak were obtained by integration, and were 46.6 joules/g for the R(+)− and 43.6 for the S(−)−limonene complexes. The heat absorption at lower temperatures (< 120°C) was not well resolved, and could have been caused by moisture evaporation as observed in the control.

The thermograms of R(+)− and S(−)−carvone complexes are given in Fig. 3. The overall profiles were similar between chiral carvone complexes and different from those of the chiral limonenes. The peak was at 140°C for both chiral carvone complexes, higher than that of chiral limonene complexes. This was expected, since carvone has a higher boiling point (230°C) than limonene (176°C) (Fenaroli, 1975). Heat absorption at lower temperature (<120°C) was also observed in the carvone complexes. Enthalpies of the peak (140°C) were 25.7 and 22.2 joules/g complex, respectively, about half those for limonene complexes. We speculated the presence of the carbonyl group in carvone probably favored hydrogen bonding with the secondary hydroxyl groups of β-cyclodextrin and led to poorer fit than that of limonene, which possessed no hydrogen bonding groups.

The difference in enthalpies at peak temperature due to chiral form for both the limonene complexes and carvone complexes was small (3.0 to 3.5 joules/g complex) suggesting there was little or no difference in fit of the two chiral forms. The significance of this difference is also questionable since enthalpy may be affected by moisture content, impurities and loading ratio (weight of flavor compound to weight of polymer matrix).

X-ray diffractometry (XRD) is normally used to characterize structure of polymers. The cell dimensions and space-group

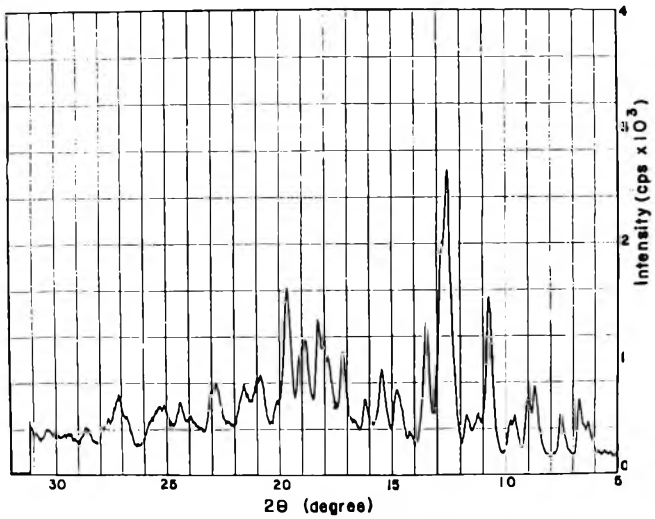
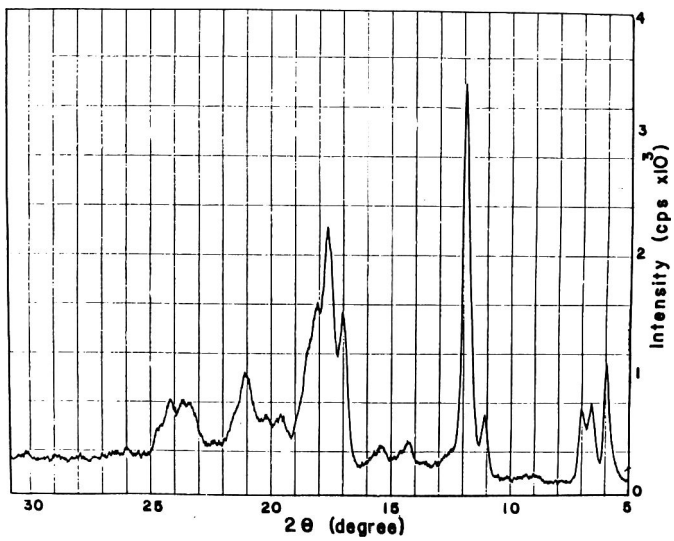


Fig. 4—X-ray diffraction pattern of pure β-cyclodextrin.



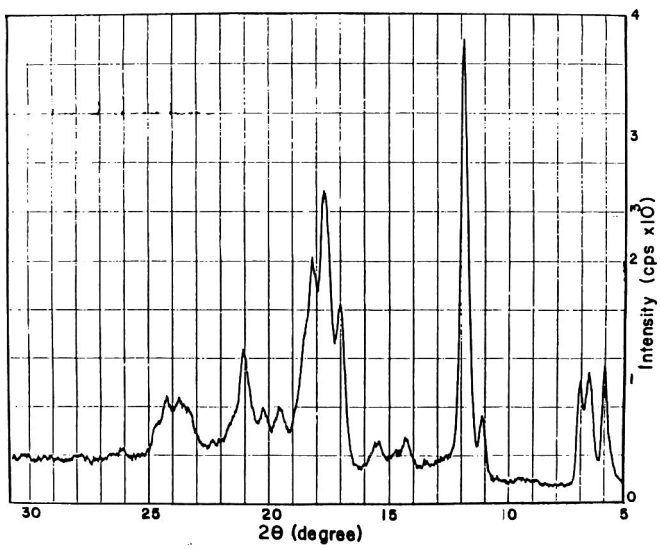
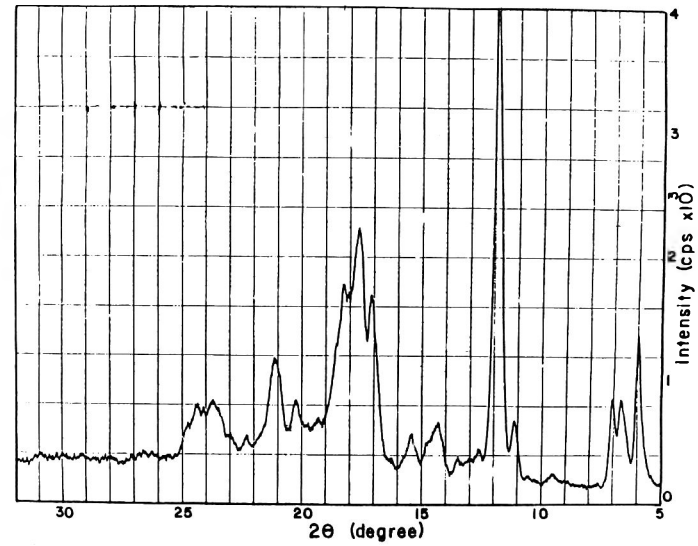


Fig. 5—X-ray diffraction patterns of chiral limonene/β-cyclodextrin complexes. [Top—R(+), Bottom—S(−)]

symmetries have been reported to reflect the packing arrangement of the torous-shaped host molecules (β-cyclodextrin), and are largely determined by the size and ionic character of



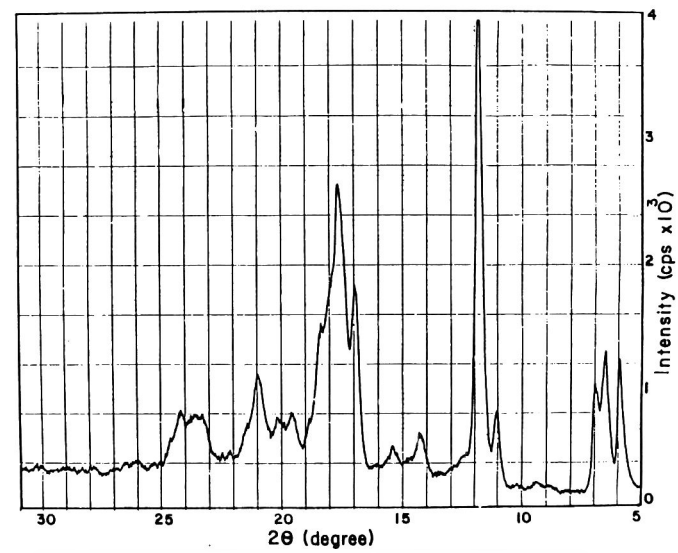


Fig. 6—X-ray diffraction patterns of chiral carvone/β-cyclodextrin complexes. [Top—R(+), Bottom—S(−)]

the guest molecules (McMullan et al., 1973). Studies of crystalline β-cyclodextrin adducts have also been reported (Hamilton et al., 1968). The detailed structure of inclusion complexes formed in our study was not determined. Therefore, only overall diffractional patterns were compared. The XRD profile of pure β-cyclodextrin is shown in Fig. 4, while those of R(+)− and S(−)−limonene inclusion complexes are shown in Fig. 5. Comparing Fig. 4 and 5, the polymeric structure of β-cyclodextrin was significantly changed after formation of the inclusion complex with the limonene molecule. The difference between profiles of the chiral limonene inclusion complexes (Fig. 5) was quite small. The only perceivable difference was at 11.8° (2 Θ); the intensity of the S(−)−limonene complex was appreciably higher than that of the R(+)−limonene complex at that angle. The same observation was seen for R(+)− and S(−)−carvone inclusion complexes (Fig. 6). The overall similarities indicated the inclusion complexes of the two enantiomeric guest molecules probably had similar overall crystalline organization. The difference in diffractional pattern at one specific angle (11.8°) was probably caused by the hydration number for the inclusion complex since the various degrees of fit between host and guest molecules have an effect on degree of hydration of the host molecule via hydrogen bonding (McMullan et al., 1973). Thus the R(+)− and S(−)− isomers apparently may differ in the degree of fit with the host

—Continued on page 1695

Electrolytic Reduction of Heme Proteins: Attempt to Prepare Stable Natural Colorant for Sausage

TATSUMI ITO, SAIKA YOSHIDA, HIROSHI KAMISOYAMA, and HISASHI TANAKA

ABSTRACT

Metmyoglobin and methemoglobin were subjected to electrolysis in presence or absence of sodium ascorbate and/or sodium nitrite. Products obtained by electrolysis were dependent on conditions. In absence of sodium ascorbate and sodium nitrite, metmyoglobin and methemoglobin were apparently transformed to red colored hydroxymet-derivatives by limited electrolysis (50 mA, 5 min) because of increased pH of electrolytes. They were reduced to oxy-derivatives by prolonged electrolysis (100 mA, ~4 hr) at neutral pH, 7.0–7.5. In presence of sodium ascorbate, met-derivatives were reduced to deoxy-derivatives by limited electrolysis (50 mA, 5 min). In presence of both sodium ascorbate and sodium nitrite, stable nitrosyl derivatives were obtained by limited electrolysis, and by prolonged electrolysis (100 mA, ~4 hr) at neutral pH values especially in presence of 1M sucrose. The electrolytically reduced products appreciably increased redness of sausages.

INTRODUCTION

MYOGLOBIN and hemoglobin are major heme-containing proteins in mammals. Hemoglobin is the oxygen and carbon dioxide carrier of the blood, while myoglobin is the oxygen storage protein of the muscle. They are the complex of apoprotein(s) (polypeptide chain) and heme(s) as prosthetic group(s). The heme is a planar tetrapyrrole porphyrin nucleus containing ferrous iron. The iron in the heme can be in ferrous or ferric state. When oxygen is present the molecule is bound reversibly and noncovalently to the sixth coordination position of the ferrous iron of heme and to the nitrogen molecule of a distal histidine residue on the opposite side of the proximal histidine residue. In the oxidized form (metmyoglobin and methemoglobin), the octahedral coordination about the iron is completed by the binding of a water molecule to the sixth coordination position. In deoxymyoglobin and deoxyhemoglobin the water molecule is absent. Since the oxidized form of heme proteins (metmyoglobin and methemoglobin with ferric iron) does not bind the oxygen molecule, the heme and the polypeptide chain provide an environment for the ferrous iron that prevents it being oxidized under usual physiological conditions (Ilan et al., 1976; Stryer, 1981; Fermi and Perutz, 1981; Dickerson and Geis, 1983).

The state of myoglobin in raw meat is changed from deoxy- to oxy- and met-forms with concomitant change of color of meat from purple red to bright red and brown respectively during postmortem storage (Fox and Thomson, 1963; Govindarajan, 1973; Giddings, 1977). The rate of changes are mainly dependent upon partial pressure of oxygen (Forrest et al., 1975). In processed meats, myoglobin in postmortem muscle reacts with nitrite in presence of ascorbate or other intrinsic reducers to form nitrosyl myoglobin during curing. The resulting nitrosyl myoglobin is converted to nitroso-myochromogen, the pinkish red pigment of cooked cured meat, upon heating (Fox and Thomson, 1963; Fox, 1966; Kramlich et al., 1973; Cassens et al., 1979). Hemoglobin in raw meat is believed subjected to similar changes during postmortem storage and processing of meat, also.

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The use of hemoglobin in animal blood as protein resources or a natural colorant has been a difficult subject in the food industry, becuase of the unusual instability of heme proteins. Myoglobin and hemoglobin are highly susceptible to oxygen and other ligands; the sixth coordination position of the ferrous iron can bind to CO and NO in addition to O_2, whereas that of the ferric iron binds to NO_2^-, OH^-, F^-, CN^-, N_3^- and H_2S (Dickerson and Geis, 1983). Metmyoglobin and methemoglobin catalyze the oxidation of lipids, resulting in development of rancidity (Tarladgis, 1961). Such undesirable properties of heme proteins have been obstacles to their use as protein resources or as natural colorants. Therefore, to suppress the susceptibility of the ferrous iron of the heme group against oxidation is one goal in the use of heme proteins in the food industry.

The oxidized form of hemin and heme proteins such as cytochrome c, myoglobin and hemoglobin can be electrochemically transformed to reduced derivatives using polarographic techniques (Gygax and Jordan, 1968; Kadish and Jordan, 1970; Besto et al., 1972; Scheller et al., 1975). Conversion of those compounds from the oxidized to reduced form is believed due to reduction of heme from ferric to ferrous state (Besto and Cover, 1972; Scheller et al., 1975). Therefore, it is worthwhile to develop a new technology to prepare a natural colorant by using an electrochemical approach. However, little research has been done on the electrolysis of heme proteins.

In our study, therefore, we tried to carry out electrolytic reduction of heme protein solutions under various conditions, to obtain a stable heme pigment which would be useful for increasing color of meat products.

MATERIALS & METHODS

Materials

Heme protein (equine skeletal muscle myoglobin and bovine hemoglobin) and ampholine were purchased from Sigma Chemical Company and Pharmacia respectively. Other reagents were of analytical grade. Pork meat for sausage manufacture was obtained from a private meat packing company.

Electrolysis of heme proteins

Myoglobin and hemoglobin were dissolved in 10 mM K-phosphate buffer (pH 5.5/7.0/7.5) with or without 5 mM sodium ascorbate and/or 5 mM sodium nitrite. The same solutions without heme proteins were used as electrolytic solutions. Electrolysis (limited electrolysis) of heme proteins was made using an apparatus shown in Fig. 1.The apparatus consisted of an electrolytic cell, anode and cathode, one layer of dialyzing tube which enclosed the heme protein and an electric power supply (V-C Stabilizer JC 1055/AE 3131, Atto Ltd). Since no difference was found in spectra of electrolytically reduced heme proteins when the electrolysis was with a platinum electrode, a silver one or a carbon electrode in preliminary experiments, both insoluble (platinum and carbon) and silver electrodes were used. Upon electrolytic reduction of heme proteins, a cathode was placed in a sample solution enclosed by a dialyzing tube (cellulose tube, Union Carbide), while in the case of oxidation reaction an anode was placed in a sample solution enclosed by a dialyzing tube. The volume of heme protein

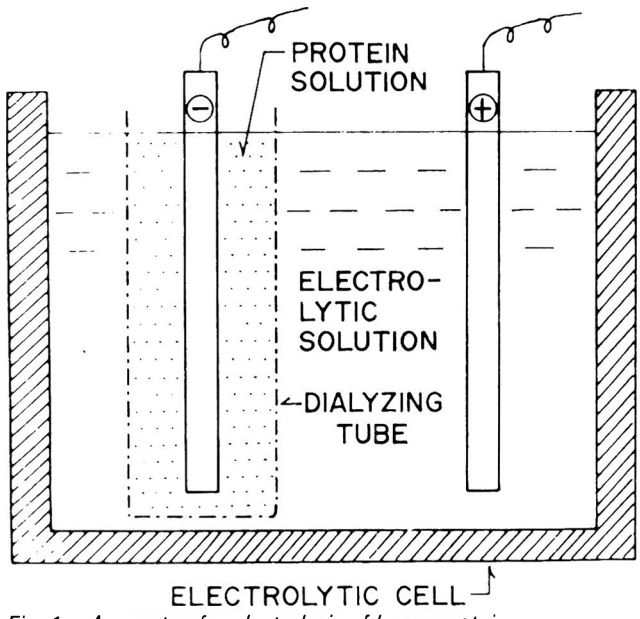

Fig. 1.—Apparatus for electrolysis of heme proteins.

samples was 10 ml and the size of the electrodes was 20 × 100 × 0.5 mm (width × height × thickness), unless otherwise mentioned. The electrolysis was at a current of 50 mA 5 min, unless otherwise mentioned.

Since pH of sample solutions increased during electrolysis, electrolysis (prolonged electrolysis) of hemoglobin in a semi-large scale (100 ml) was also carried out by titrating the pH of heme protein solution in a dialyzing tube with 25% lactic acid with or without 1M sucrose. Carbon electrodes (10 × 300 mm, diameter × length) were used, and the sample solution in a dialyzing tube was stirred with a motor drive stirrer during electrolysis. In this case, the temperature of electrolytic solutions was kept constant (about 20–25°C) by circulating cold water in a vinyl tube submerged in the electrolytic cell.

Absorption spectra of heme proteins

Absorption spectra of non-treated and electrolytically reduced heme proteins were scanned with a Hitachi 557 Double Wave Length Double Beam Spectrophotometer at a speed of 120 nm/min.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed on 7.5–17.5% acrylamide gradient slab gels according to the procedure of Laemmli (1970).

Gel electrofocusing of nontreated and electrolytically reduced heme proteins

Five percent polyacrylamide slab gel (crosslinking, 3%; gel size, 110 × 110 × 0.5 mm) containing 20% glycerol and 10% ampholine (pH 5.8–8.0) were used. After the preparation of gels, pre-run was done at 5W electric power at 2°C without application of samples in order to set the pH gradient of the gels. Fifty μg of heme protein samples were applied on the gels. Electrofocusing of heme proteins was at 10 W electric power 1 hr using an electrofocusing apparatus (Resolmax R-IEF, Atto Ltd) and high voltage (maximum 3000 V) electric power (AE-3122, Atto Ltd.). Buffer system for electrofocusing was 0.4 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-0.1 M NaOH. Gels during electrofocusing were kept at constant 2°C by circulating ice-cold water under the gels.

After electrofocusing, one edge of the gels was cut off and divided into 5 × 10 × 0.5 mm pieces, and the pieces suspended in 0.5 mL distilled water. The pH of the resulting suspensions was examined with a Hitachi-Horiba pH meter $F\text{-}7_{II}$ for examining pH gradient of the gels. The remaining gels were fixed with a large volume of a mixture of 5% sulfosalicylic acid and 10% trichloroacetic acid for 30 min, followed by soaking the gels in a destaining solution (methanol:acetic acid:distilled water = 3:1:6) for 30 min to substitute the fixing solution in the gels with the destaining solution. Gels were staining with 0.2% Coomassie Brilliant Blue R-250 dissolved in a mixture of 30% methanol and 10% acetic acid 2 hr at room temperature. Destaining was made with the destaining solution described above.

Chemical oxidation of electrolytically reduced heme proteins

Electrolytically reduced heme proteins obtained in presence or absence of sodium ascorbate and/or sodium nitrite were oxidized by addition of 1/10 vol of 0.05M/0.1M potassium ferricyanide. The change of spectrum of the mixtures was examined with the spectrophotometer described above. After 10 min incubation, mixtures of electrolytically reduced myoglobin/hemoglobin and potassium ferricyanide were subjected to gel filtration on a Sephadex G-50 column (1.5 × 87 cm) to separate met-derivative of the heme proteins from the mixtures (Geyer and Lemberg, 1971). Elution was with 10 mM K-phosphate buffer (pH 7.0) or with 0.05 M Glycine-NaOH buffer (pH 12.0), and 1.5 ml fractions were collected with a fraction collector (LKB 2211 Superrac). The spectrum (250–700 nm) of resulting eluates was obtained with the spectrophotometer. For detection of ferrocyanide, the development of blue color (λ_{max} = 710 nm) of each fraction was examined by dropping 50 μl of 0.1 M $FeCl_3$.

Protein concentration

Protein concentration was determined by the biuret method (Gornall et al., 1951), which had been standardized with bovine serum albumin.

Preparation of experimental sausages

Lean portion of pork meat (Longissimus thoracis) was chopped with a meat chopper and the resulting ground meat was cured either with a mixture of 2% sodium chloride, 0.1% sodium pyrophosphate, 150 ppm sodium nitrite and 550 ppm sodium ascorbate, or with another mixture of 2% sodium chloride and 0.1% sodium pyrophosphate, for 24 hr in a cold room (3–5°C). To 100g of the cured pork meat, 10 ml of cold nontreated or electrolytically reduced hemoglobin was added for the experiment, and for a control experiment 10 mL of cold nontreated or electrolyzed buffer solution without hemoglobin was added. After mixing well with a spatula about 5 min, the mixtures were stuffed into polyvinyliden chloride casings, followed by cooking in a water bath at 75°C for 45 min. Then, the sausages were cooled in a cold room (3–5°C) for 24 hr.

Hunter value of experimental sausages

After cooling, the sausages were sliced into pieces of 8.0 mm thickness. Hunter values (L, a and b) of the sliced sausages were examined with a color and color difference meter (ND-K6B, Nihon Denshoku Ltd).

RESULTS & DISCUSSION

AS IS WELL KNOWN, metmyoglobin has absorption maxima at about 500 and 630 nm, while deoxy-myoglobin has a single absorption maximum at 555 nm. Oxymyoglobin and nitrosyl myoglobin have two peaks in their absorption spectra at 544 and 582 nm, and at 545 and 575 nm, respectively (Encyclopaedia Chimica, 1962; Fox and Thompson, 1963; Kramlich et al., 1973). Figure 2 shows absorption spectra of heme proteins before and after limited electrolysis. The spectrum of metmyoglobin was changed to an oxymyoglobin-like spectrum after the limited electrolysis at 50 mA for 5 min, i.e., the absorption maxima of the electrolytically reduced product were about 544 and 582 nm (Fig. 2A). Likewise, methemoglobin was changed to an oxyhemoglobin-like derivative by electrolysis (50 mA, 5 min) (Fig. 2b).

In the case of the limited electrolytic reduction described above, however, pH of heme protein solutions increased with increasing electric current during the electrolysis. For example, pH value of myoglobin solution in a dialyzing tube increased to about 12 after electrolysis at 50 mA for 5 min (Fig. 3). The

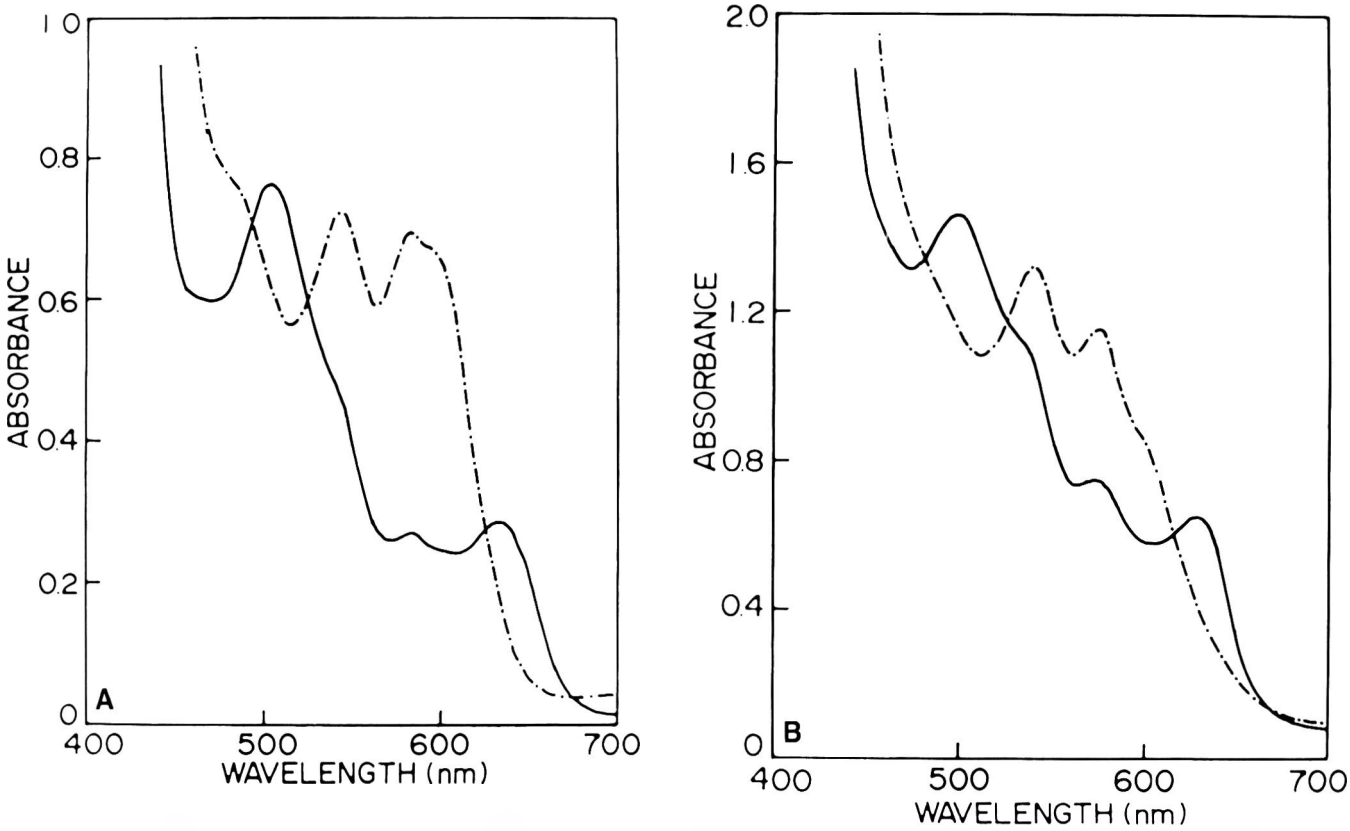

Fig. 2.—Absorption spectra of heme proteins before and after electrolysis: (A) Absorption spectra of myoglobin (0.1 mM); (B) absorption spectra of hemoglobin (0.05 mM); ————before limited electrolysis (50 mA, 5 min); — • — after limited electrolysis.

increase in pH of electrolytes was probably due to the accumulation of OH^- . To examine whether the change of color of met-derivative during electrolysis was due to electrolysis of heme proteins or increase in pH, we investigated effect of an alkaline titration on color of myoglobin. Quite similar absorptoin spectra to that of Figure 2 and those of Austin and Drabkin (1935) were obtained simply by titrating sample solutions with an alkaline solution (1.0 M NaOH). In addition, the change of absorption spectra of heme proteins obtained by alkali titration was reversed to that of met-derivatives by titrating with 1M HCl (Data not shown). Therefore, development of the color of met-drivatives during limited electrolysis was mostly due to increase in pH. Although absorption spectra of Fig. 2 were somewhat different from methemoglobin at alkaline pH shown by Austin and Drabkin (1935), a characteristic feature of absorption spectra at around 590–600 nm (shoulder) indicates a major component of the products obtained by limited electrolysis (50 mA, 5 min) might be alkaline heme proteins. Hydroxymet-heme proteins (The alkaline form of ferric heme proteins) is characterized by the sixth ligand of the heme iron: OH^- bound to the sixth coordination position of the heme (Okazaki et al., 1976).

Figure 4 shows the effect of limited electrolysis on the spectrum of chemically reduced myoglobin. In this experiment, metmyoglobin was pre-incubated with 50-fold molar concentration of sodium ascorbate 1 hr at 25°C prior to electrolysis. The mixture of metmyoglobin and sodium ascorbate was subjected to limited electrolysis (50 mA, 5 min). The spectrum of metmyoglobin was changed to that of oxymyoglobin during the incubation of metmyoglobin with ascorbate, and then it was further changed to that of deoxymyoglobin-like derivative having a single absorption maximum at about 555 nm by limited electrolysis, although the pH of electrolyte increased to about 12. This suggests the e^-_{aq} release oxygen molecule from oxymyoglobin and the released oxygen as well as oxygen molecules dissolved in the mixture might be incorporated into oxygen scavenger ascorbate (Ilan et al., 1976). In the case of hemoglobin, however, absorption spectrum of methemoglobin was changed to that of oxyhemoglobin during the incubation of methemoglobin with 5 mM sodium ascorbate for about 1 hr. Then it was gradually changed to that of deoxyhemoglobin during prolonged incubation, so that there was no need to electrolyze in order to get deoxyhemoglobin (data not shown). These results suggest that a main reason for the difference in the effect of chemical reducer (ascorbate) between myoglobin and hemoglobin is due to the allosteric effect of hemoglobin (Monod et al., 1963).

Figure 5 shows the effect of limited electrolysis on the spectrum of myoglobin and hemoglobin with or without sodium ascorbate and sodium nitrite (5 mM). The spectrum of metmyoglobin was changed to have two absorption maxima at about 545 and 575 nm after incubation of metmyoglobin with sodium ascorbate and sodium nitrite at pH 7.0. This spectrum is quite similar to that of nitrosyl myoglobin (Fox and Thompson, 1963), indicating its formation during incubation. Then, the spectrum of the mixture of myoglobin, ascorbate and nitrite was changed to the one having absorption maximum at around 546 nm and a shoulder at around 570 nm after the limited electrolysis (50 mA, 5 min). In that case, the pH of electrolyte increased up to about pH 12. Finally, the spectrum of myoglobin was changed to the one having absorption maximum at about 555 nm after slightly prolonged electrolysis (50 mA, 10 min or 100 mA, 5 min). This indicates that slightly prolonged electrolysis of myoglobin following incubation with sodium ascorbate and sodium nitrite produced a deoxymyoglobin-like derivative (Fig. 5A). This result also suggests that NO molecule relased from the heme might be incorporated into ascorbate. In the case of methemoglobin, quite similar spectra were obtained after incubation of methemoglobin with sodium ascorbate and sodium nitrite 1 hr, and after limited electrolysis of the mixture of methemoglobin, ascorbate and nitrite (Fig. 5B).

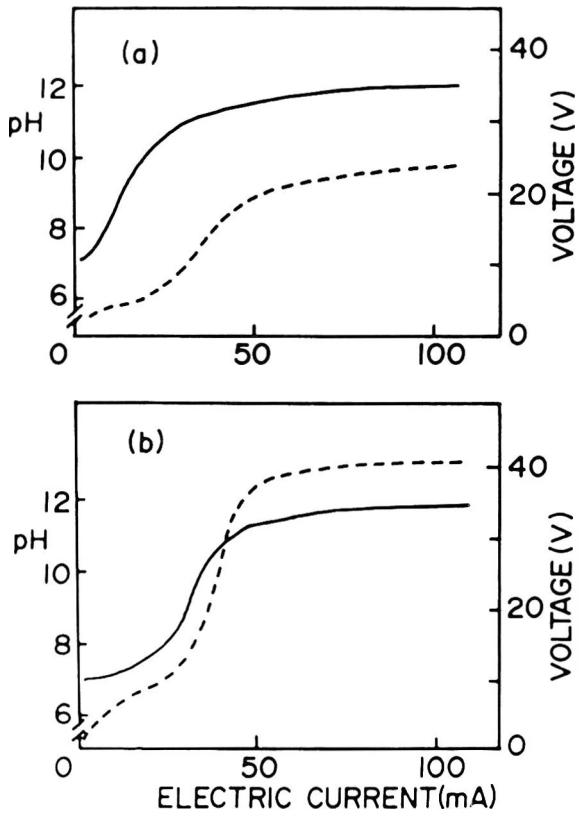

Fig. 3.—Profile of pH change of electrolytes during electrolysis: (a) Myoglobin (0.1 mM) in 10 mM K-phosphate buffer (pH 7.0); ———profile of pH change; ----- profile of voltage change. (b) Hemoglobin (0.05 mM) in 10 mM K-phosphate buffer (pH 7.0): indications are the same as in (a).

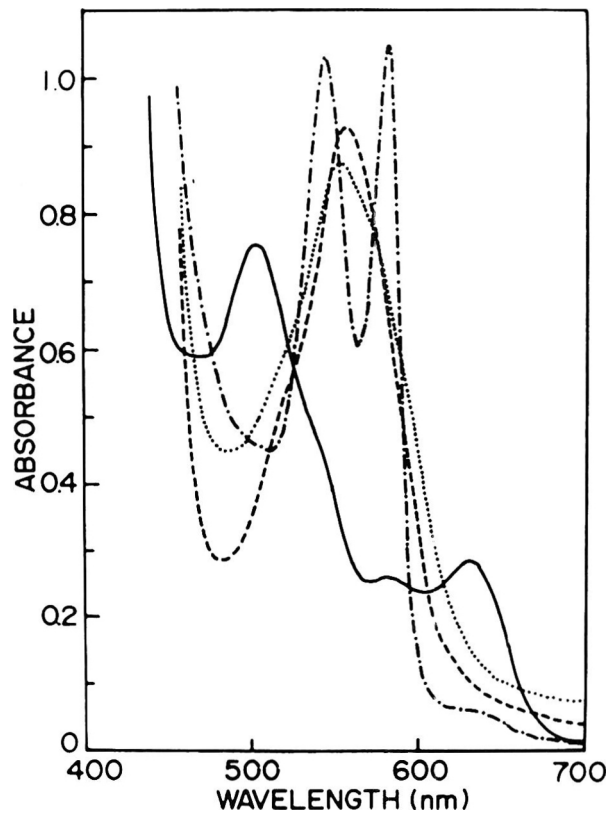

Fig. 4.—Effect of limited electrolysis on the spectrum of myoglobin following incubation with sodium ascorbate: ——— before incubation; — • — after 60 min incubation; ••••• after limited electrolysis; ----- after addition of 5 mM sodium hydrosulfite. Concentration of myoglobin was 0.1 mM.

In spite of the influence of electrolytic reduction on spectral features of heme proteins, as described above, no structural change was observed in SDS polyacrylamide gel electrophoretograms of the heme proteins after the limited electrolysis (data not shown). This indicates that limited electrolysis at 50 mA for 5 min has no drastic effect on structure of heme proteins. Isoelectric focusing patterns of myoglobin demonstrated that the myoglobin sample used contained three major subcomponents and several minor ones. The isoelectric points of the three major subcomponents were about pH 7.2, 6.8 and 6.6. These values were almost the same as those of Satterlee and Snyder for bovine skeletal myoglobin (1969). In addition, no electrolytic reduction effect was observed in the isoelectric focusing pattern of myoglobin, while with chemical treatment of myoglobin with sodium ascorbate and sodium nitrite intensity of the band of pH 6.6 increased (Fig. 6). This may have been due to deamination of amides in myoglobin (Vesterberg, 1967; Satterlee and Snyder, 1969). However, this result suggests that limited electrolytic reduction treatment has almost no effect on the net charge of the globin moiety of myoglobin. There was also no appreciable effect of electrolytic reduction on the isoelectric focusing pattern of hemoglobin, although the pattern of isoelectrolytic focusing of hemoglobin was not so clear as with myoglobin (Data not shown). Of course, the change of the spectrum solely dependent on electrolytic reduction of heme proteins was also observed when myoglobin was subjected to prolonged electrolysis (100 mA, ~4 hr) by keeping the pH of the sample solution constant (pH 7.0–7.5) with 25% lactic acid titration during electrolysis. As shown in Fig. 7, the spectrum of methemoglobin was changed to be similar to that of Fig. 2B. However, absorbance of the spectrum decreased during electrolysis probably because of the decrease in solubility of the electrolyzed sample. In addition, a much longer time was required to reduce methemoglobin than in the case of the experiment shown in Fig. 2B. Since Ilan et al. (1976) demonstrated that, in a solution system of metmyoglobin (or methemoglobin) containing oxygen, e^-_{aq} radical reduced the heme group of metmyoglobin (or methemoglobin) and then the resulting reduced heme protein molecule bound with oxygen during the reduction reaction, this result suggested the ferri-state of the heme moiety of methemoglobin molecule was reduced to the oxygenated (ferrous) state by prolonged electrolysis. At the same time the water molecule bound to the sixth coordination position of the ferric iron was replaced by oxygen molecule dissolved in the reaction medium.

Figure 8 shows the effect of prolonged electrolysis at constant pH, 7.5 on the spectrum of heme protein in the presence of 1M sucrose. The spectrum of hemoglobin was clearly changed to that of a ferrous derivative and there was no remarkable decrease in absorbance of the sample during electrolysis. There was also no increase in turbidity of the sample. This result indicates hemoglobin was protected from denaturation by sucrose during electrolysis. And hence, this result suggests electrolytically reduced heme proteins could be successfully obtained by electrolysis at neutral pH in presence of sucrose.

Geyer and Lemberg (1971) have reported that cyanide ion resulting from breakdown of ferricynide complex, forms cyanoheme pigment when ferricyanide is used to oxidize heme proteins. We separated heme proteins from the mixture of electrollytically reduced hemoglobin and potassium ferricyanide by gel filtration. Electrolytically reduced-potassium ferricyanide treated hemoglobin eluted first, followed by detection of ferrocyanide ion, and finally potassium ferricyanide (Fig. 9A). The resulting heme protein had a similar spectrum to those of Fig. 2B, 7 and 8 (Fig. 9B). This suggests electrolytically reduced heme proteins were resistant against chemical oxidation

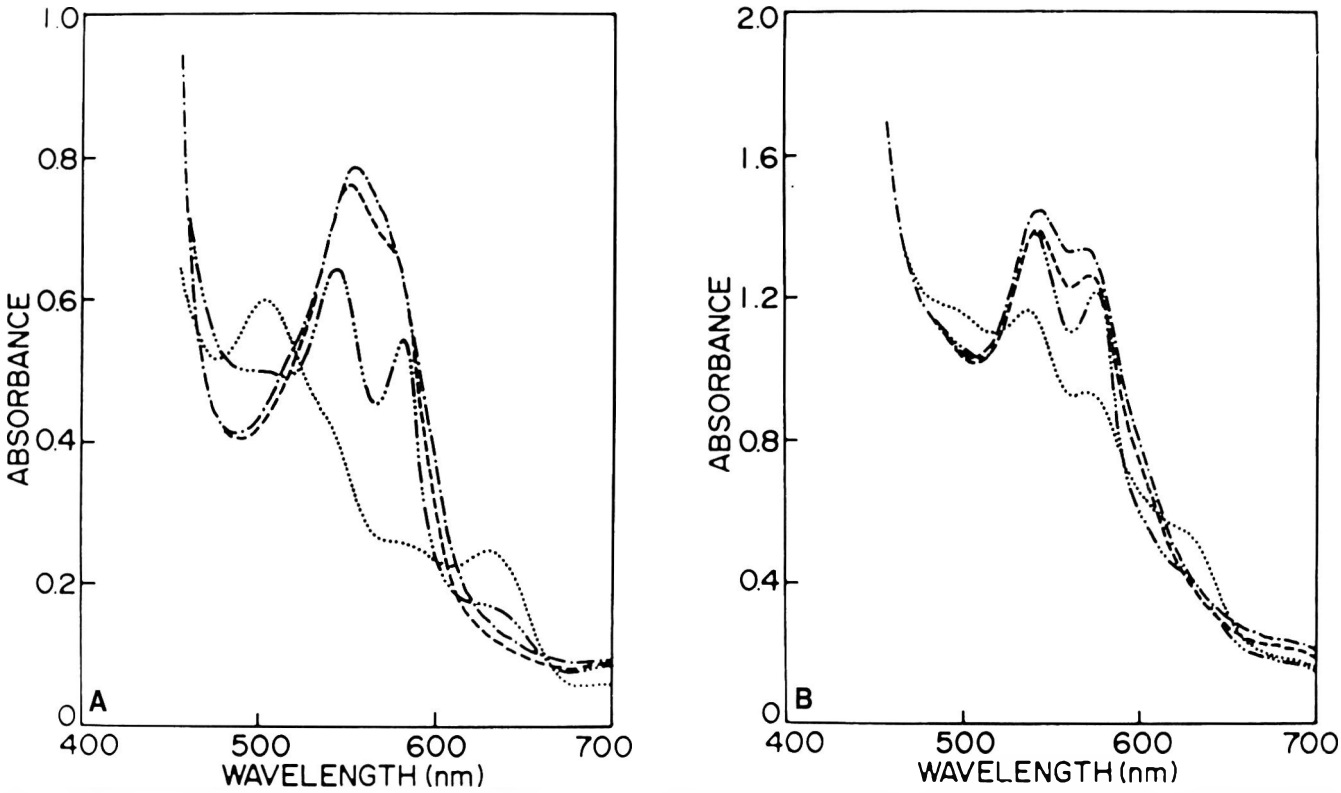

Fig. 5.—Effect of limited electrolysis on the spectra of heme proteins following the incubation with sodium ascorbate and sodium nitrite (A) Myoglobin (0.1 mM): ••••• before incubation; —•••— after incubation; ----- after limited electrolysis (50 mA, 5 min); — • — after slightly prolonged electrolysis (100 mA, 5 min). (B) Hemoglobin (0.05 mM): all indications are the same as in (A).

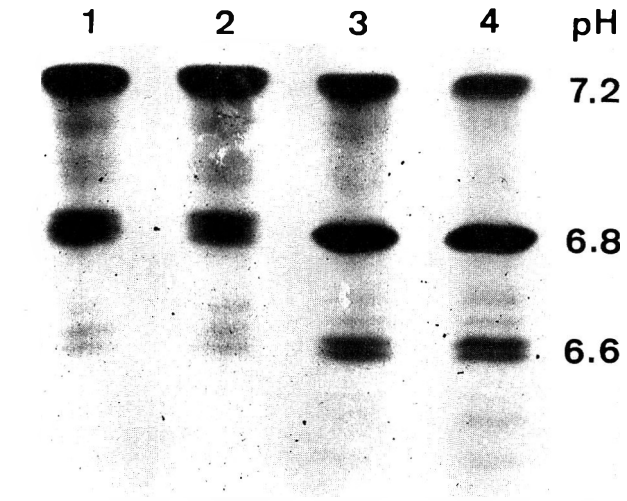

Fig. 6.—Isoelectric focusing patterns of myoglobin: (1) Non-treated myoglobin; (2) electrolyzed myoglobin; (3) myoglobin incubated with 5 mM sodium ascorbate and sodium nitrite; (4) electrolyzed myoglobin following incubation with 5 mM sodium ascorbate and 5 mM sodium nitrite. About 50 μg of sample was loaded on each slot of the gel.

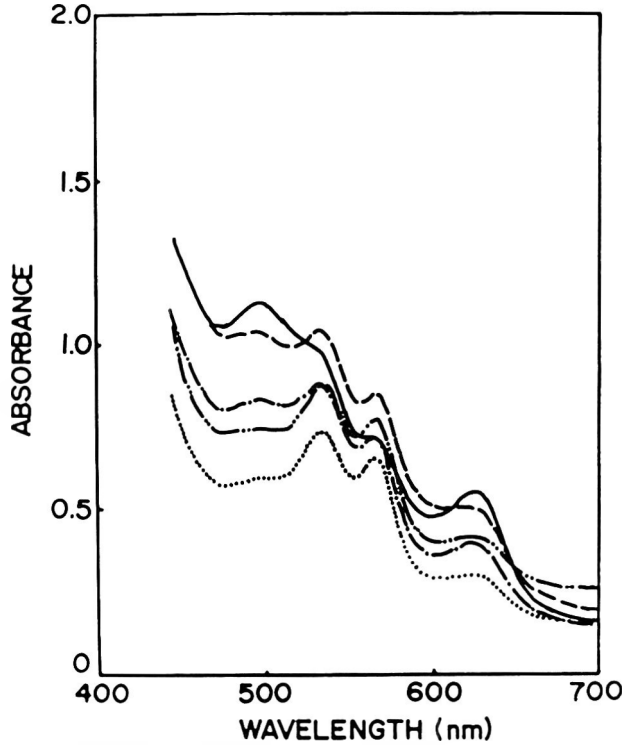

Fig. 7.—Change of spectrum of hemoglobin during prolonged electrolysis at a neutral pH: ———spectrum after 60 min electrolysis; ----- spectrum after 120 min electrolysis; — • — spectrum after 180 min electrolysis; —••— spectrum after 250 min electrolysis; ••••• spectrum after 330 min electrolysis. Concentration of hemoglobin was 0.05 mM.

reaction of potassium ferricyanide. With electrolytically reduced hemoglobin subjected to electrolysis for an extremely prolonged period (more than 4 hr), however, the resistance was appreciably reduced (data not shown).

To examine availability of electrolytically reduced hemoglobin in meat processing as a colorant, we prepared experimental sausages with or without addition of electrolytically reduced hemoglobin. Although the color of control sausages varies considerably sample to sample, as is represented in Table 1, redness (a value) of the sausages appreciably increased after the addition of electrolytically reduced hemoglobin (pre-

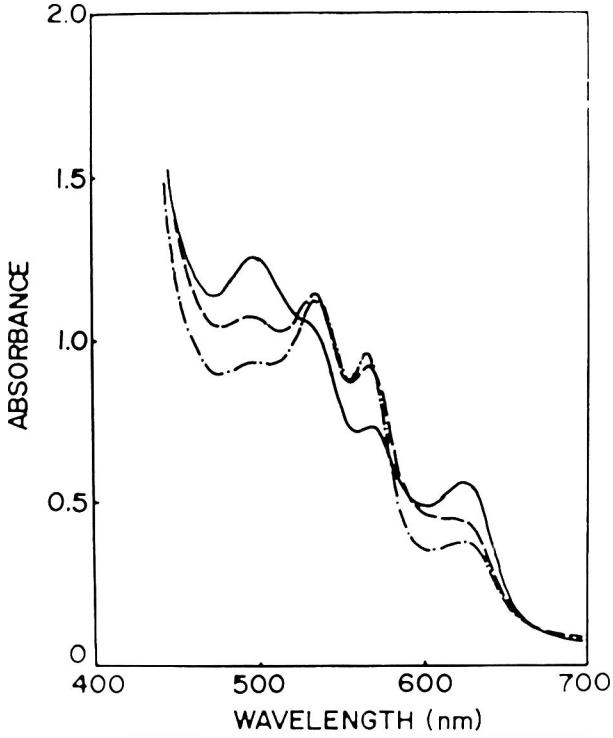

Fig. 8.—Effect of sucrose on the spectrum of hemoglobin during prolonged electrolysis: ———spectrum after 60 min electrolysis; ----- spectrum after 120 min electrolysis; — • — spectrum after 240 min electrolysis. Concentration of hemoglobin was 0.05 mM.

pared by limited electrolysis at 50 mA for 5 min) which had been treated with sodium ascorbate and sodium nitrite. Therefore, electrolytically reduced heme proteins obtained by the limited electrolytic reduction treatment may be useful for sausage manufacture even if the electrolyzed product is hydroxymet-hemoglobin. Experimental sausages containing electrolytically reduced hemoglobin which had been electrolytically reduced in presence of sodium ascorbate, sodium nitrite and sucrose also showed appreciably higher a values than other sausages which had been subjected to different treatments (Table 2). The color of meat products was dependent on the content of heme proteins as well as the redox potential of meat (Taylor and Walters, 1967; Fox and Ackerman, 1968; Mohler, 1974). Therefore, it is difficult to control color of meat simply by adding heme proteins. Taking the present result (Table 1) into consideration, however, we can speculate that electrolytic reduction of heme proteins in presence of sodium ascorbate, sodium nitrite and sucrose might produce a relatively stable meat colorant. However, the disadvantage of this procedure is the consumption of much electric power for preparing electrolytically reduced heme proteins.

As described, we tried to prepare two types of electrolytically reduced heme proteins. One was hydroxymet-derivatives obtained in media with alkaline pH produced by limited electrolysis of heme protein with or without sodium ascorbate and/or sodium nitrite. The other type was a ferroderivative of hemoglobin produced by prolonged electrolysis at neutral pH. Alkaline treatment of heme proteins may provide one step to develop a useful procedure in utilizing heme proteins in the food industry.

Although the present products obtained by electroysis of heme proteins were not necessarily a satisfactory meat colorant with overall desirable properties as a natural colorant, our results suggest a possibility of success for production of electrolytically reduced derivatives of heme proteins useful in the meat industry for increasing color. In addition, electrolysis may provide a procedure for producing nitrosyl derivatives of heme proteins with reduced levels of sodium nitrite.

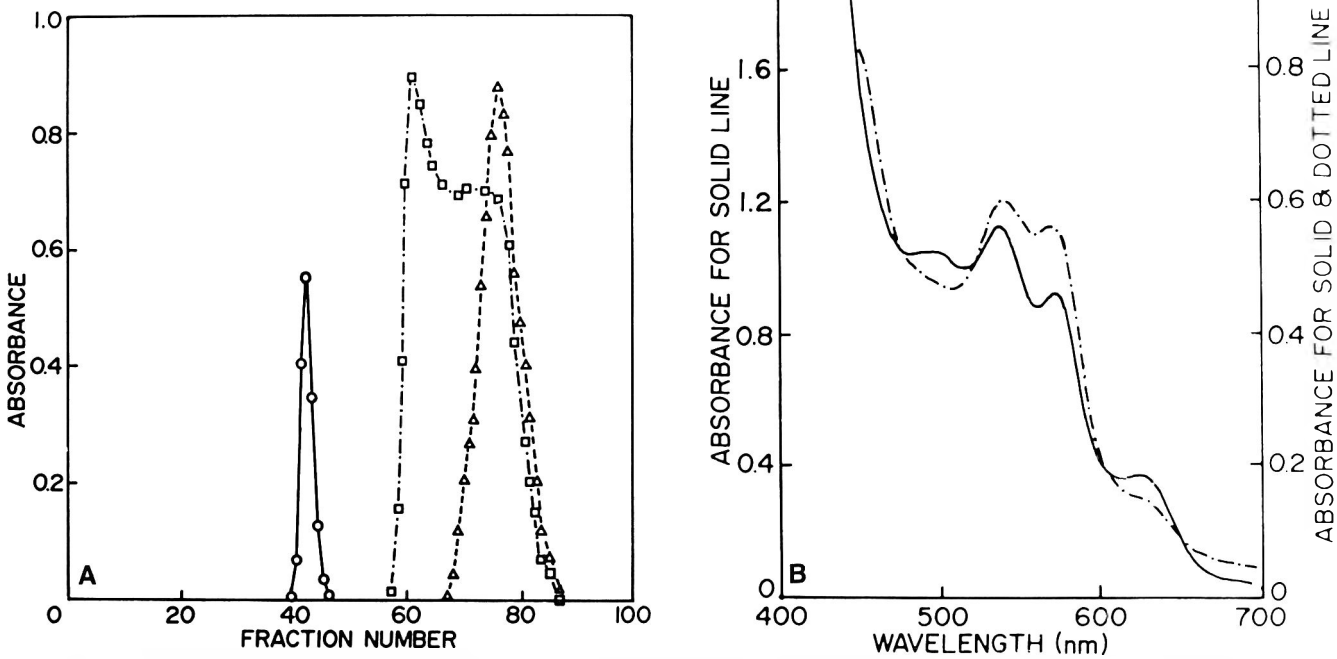

Fig. 9.—Effect of ferrocyanide ion elimination on the spectrum of electrolytically reduced-potassium ferricyanide treated hemoglobin: (A) Elution pattern of potassium ferricyanide treated-electrolytically reduced hemoglobin. Elution was conducted with 10 mM K-phosphate buffer (pH 7.0). ○—○ Electrolytically reduced-potassium ferricyanide treated hemoglobin (absorbance at 540 nm); □—•—□ ferrocyanide ion (absorbance at 710 nm); △---△ potassium ferricyanide (absorbance at 420 nm). (B) Spectra of electrolytically reduced hemoglobin before and after gel filtration. ———Mixture of electrolytically reduced hemoglobin and potassium ferricyanide; — • — electrolytically reduced-potassium ferricyanide treated hemoglobin after gel filtration. In this experiment, electrolytic reduction was carried out for 2 hr in the presence of 1M sucrose.

Table 1—Color values of experimental sausages: Effect of limited electrolytically reduced hemoglobin on color development of experimental sausages[a]

Hunter value	Sample					
	C-1	E-1	C-2	E-2	C-3	E-3
L	68.2	62.8	70.3	64.1	67.9	63.9
a	7.8	11.2	3.5	4.4	7.3	9.5
b	10.4	9.4	10.1	10.5	8.9	8.8

[a] C-1 (Control-1), pork meat cured with 2% NaCl, 0.1% sodium pyrophosphate, 150 ppm sodium nitrite and 550 ppm sodium ascorbate; E-1 (Experiment-1), to Control-1, added 0.064% (wt/wt) limited electrolytically reduced hemoglobin which had been incubated with 5 mM sodium ascorbate and 5 mM sodium nitrite; C-2, pork meat cured with 2% sodium chloride and 0.1% sodium pyrophosphate; E-2, to Control-2, added 0.064% (wt/wt) non-treated hemoglobin; C-3, pork meat cured with 2% sodium chloride and 0.1% sodium pyrophosphate; E-3, to Control-3, added 0.064% (wt/wt) limited electrolytically reduced hemoglobin which had not been incubated with ascorbate and nitrite. Means of five determinations.

Table 2—Color values of experimental sausages: Effect of prolonged electrolytically reduced hemoglobin on color development of experimental sausages[a]

Hunter value	Sample				
	I	II	III	IV	V
L	61.5	62.5	64.5	64.2	69.1
a	10.5	17.0	6.0	6.2	5.4
b	9.2	9.0	9.6	9.6	9.5

[a] Sample I, To 100 g cured meat, added 0.064% (wt/wt) prolonged (100 mA, 4 hr) electrolytically reduced hemoglobin (see Fig. 7); Sample II, to 100 g cured meat, added 0.064% (wt/wt) prolonged (100 mA, 2 hr) electrolytically reduced hemoglobin as in Fig. 8 (Pork meat in Samples I and II was cured with 2% NaCl, 0.1% sodium pyrophosphate, 150 ppm sodium nitrite and 550 ppm sodium ascorbate); Sample III, to 100 g cured meat, added 0.064% (wt/wt) prolonged (100 mA, 2 hr) electrolytically reduced hemoglobin without the preincubation with sodium ascorbate and sodium nitrite; Sample IV, to 100 g cured meat, added 0.064% (wt/wt) non-treated hemoglobin; Sample V, to 100 g cured meat, added 10 ml, 10 mMK-phosphate buffer (pH 7.0). (Pork meat in Samples III, IV and V was cured with 2% NaCl and 0.1% sodium pyrophosphate). Means of five determinations.

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*CYCLODEXTRIN INTERACTION. . .*From page 1688

molecule. This may explain the slight difference in heat absorbed for vaporization between enantiomeric compounds from their corresponding inclusion complexes.

The comparison of Fig. 5 and 6 also illustrates that carvone complexes differed in diffractional intensity at 5° to 7°, 11.8° and 16.5° to 19° from limonene complexes to a certain extent. These differences indicate that β-cyclodextrin complexes of carvone and limonene differed in crystalline organization. This agrees with the appreciable difference observed earlier between their corresponding enthalpies of dissociation.

CONCLUSION

DSC ANALYSES demonstrate that the difference in heat absorption (enthalpy) between β-cyclodextrin inclusion complexes of R(+)− and S(−)−limonenes at peak temperature (130°C) was slight and of questionable significance (3.0 joules/g complex). A similar finding was observed between complexes of chiral isomers of carvone at 140°C. A comparison of X-ray diffraction patterns of both chiral limonene and chiral carvone complexes showed that chiral form had a slight influence on pattern when 2 Θ was at 11.8°. Otherwise the overall diffraction patterns were very similar between the chiral complexes.

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Time-Temperature Equivalence of Discrete Particles during Thermal Processing

F. SADEGHI and K. R. SWARTZEL

ABSTRACT

A method is developed to determine time-temperature equivalencies within discrete particles during thermal processing. Encapsulated calibration materials were examined for establishing the equivalent point for a canning process where results were in good agreement with thermal evaluations using Ecklund thermocouple data. Process characteristics for continuous flow, as Reynolds number, Nusselt number, residence time distribution within heat exchangers and holding tubes, and size and shape of particles are considered for expanding the use of the methodology.

INTRODUCTION

THE DEVELOPMENT of aseptic processing and packaging technology for food products containing particulates has created considerable interest in the food processing industry due to its potential for reduction of packaging costs, its economical and efficient means of microbial inactivation, and its improvements in product quality. In order to achieve these benefits, the thermal processes must ensure adequate sterilization for each particle. To provide this product safety, evaluation of thermal processes is essential; however, accurate measurement of temperatures within particles is very difficult. For this reason, mathematical modeling or indirect chemical or microbial indicators have been used to define such thermal systems.

A few researchers have investigated the possibility of predicting the temperature of the particle center via mathematical modeling. de Ruyter and Brunet (1973) developed a mathematical model for spherical particles processed in a scraped surface heat exchanger (SSHE). This model assumed infinite value for the conductive heat transfer coefficient at the particle surface. Normally this assumption would overestimate the lethal effect within the particle. However, according to the authors, significant over-sterilization of the liquid phase occurred when attempting to achieve sterility at the center of particles larger than 3.2 mm. They explained that the food behaved as a homogeneous material for particles up to 3.2 mm. The significance of the residence time distribution was not reported by these authors.

Manson and Cullen (1974) introduced a model for cylindrical particles in a SSHE. They assumed infinite heat transfer coefficient and demonstrated the relative importance of residence time distribution in process design. They demonstrated that significant underprocessing occurred if plug-flow conditions were assumed for heterogeneous foods processed in a SSHE. Holding tube length would be computed on the basis of laminar flow or as a suitable multiple of holding tube length computed on the basis of turbulent flow. The exact value of the multiple would depend on the proportion of the particle matter in the food and the thermal properties of the particulate matter.

Dail (1985) developed analytical solutions for cubic and cylindrical particles in a SSHE and substituted into Ball's formula to calculate required hold time. He noted the limitations of those formulas in aseptic processing. Lethality integration in Ball's formula method starts below the lethal range, the assumption that f_c (temperature response parameter, cooling) is equal of f_h (temperature response parameter, heating), and the determination of f_h and subsequently thermal diffusivity results from the fluid carrier.

Sastry (1986) developed a mathematical model for aseptic processing of foods with particulates in a SSHE applicable to particles of regular and irregular shapes. This model considered the influence of particle size, residence time distributions and estimated values of convective coefficients. This model also assumed that viscous dissipation was negligible, all particles collectively acted as a population of particles of equal size, liquid media was perfectly mixed in the radial direction of the heat exchanger, and the moving particles remained at the same average speed as the fluid. This model was tested for various conditions but was not validated using experimental data. Lack of data does not allow conclusions concerning validity of that model.

Swientek (1987) described an aseptic process simulator (designed and built by Cherry-Burrel Corp., Cedar Rapids, IA.) for performing heat penetration profiles on particulate products. The simulation system consisted of a particulate suspension chamber, assembled with temperature probes, tubular heat exchanger, positive displacement pump with variable speed drive, flowmeter, and a data logger. He further explained that the aseptic process simulator provided accurate monitoring of continuous aseptic processing conditions during heat up, hold, and cool down. The simulator was designed to handle various particle sizes and types, flow rates, product carriers, and heat up and cool down rates. The National Food Processors Association (NFPA) has been working on a computer-based mathematical model of aseptic particulate processing. With the simulator, NFPA will be able to obtain data for verifying or validating the model. Moreover, that will allow determination of actual heating rate constants, which can be used to refine the model. The equipment does not consider the shear stress effect at the wall, changes in shape of particles (damage), and thermal conductivity variations between particles.

Several studies have dealt with microbial spores as an indirect indicator of aseptic thermal processes. *Bacillus anthreacis* suspended in polymethylmethacrylate ("perspex"), in spherical shapes, was used by Hunter (1972) to determine the heat transfer coefficient between liquid (water) and particles in a SSHE. Also Heppell (1985) measured the convective heat transfer coefficient at the boundary between a heated liquid and a sphere particle via immobilizing *Bacillus stearothermophilus* TH 24 in calcium alginate. These models assumed an instantaneous fluid temperature rise along with utilization of batch generated kinetic data in continuous processing conditions, where the accuracy of the latter assumption has been questioned (Sadeghi and Swartzel, 1990). A number of empirical relationships are available for estimation of the surface heat transfer coefficient (Nusselt number correlations) for a SSHE (Skelland et al., 1962; Weisser, 1972; Trommelen, 1970; Sykora and Navratil, 1966; Sykora et al., 1968), although almost none are available for non-Newtonian foods. Nusselt number correlations for heterogeneous foods processed in SSHE

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are not available in the literature and those for homogeneous foods are often used. This must be done with caution since experimental conditions under which such relationships are developed may not match the situation under consideration.

Swartzel (1982, 1986) developed a novel thermal evaluation technique, the Equivalent Point Method (EPM), which describes thermal systems with one equivalent time and temperature (t_E, T_E), independent of activation energy (E_a). For any time-temperature profile [T(t)], the EPM is based upon the integration of the n^{th} order rate law equation for concentration (C) change ($-dC_i/dt = k_i C_i^n$) with substitution of the Arrhenius equation, $k_i = A_i \exp\left(-\frac{Ea_i}{R\,T(t)}\right)$ yielding:

$$G_i = \frac{M_i}{A_i} = \int_0^{t_F} \exp\left(-\frac{Ea_i}{R\,T(t)}\right) dt \qquad (1)$$

where Ea_i and A_i are the Arrhenius activation energy and frequency constant for constituent i, R universal gas constant, G_i is the thermal reduction relationship, and M_i is defined by Swartzel and Jones (1984):

$$M_i = -\int_{C_{i,0}}^{C_{i,f}} \frac{dC_i}{C_i^n} \qquad (2)$$

In order to evaluate t_E and T_E, at least two pairs of Ea_i and G_i values must be known. Then, either the Line Intersections (LI) method (Swartzel, 1982; Sadeghi et al., 1986) or the Weighted Least Squares Linear Regression (WLSLR) method (Nunes and Swartzel, 1990) can be used for parameter estimation. According to equation (1), G-values can be evaluated using either the time-temperature profile or concentration changes (M_i/A_i). Sadeghi and Swartzel (1990) demonstrated, by utilizing the EPM in reverse, constituent changes of the three calibration materials can be used to determine the system's equivalent point. Ea_i and A_i for selected calibration materials were determined. They explained that knowledge of the kinetic parameters along with initial and final concentrations of calibrating materials are required to determine the time-temperature equivalent point for any system without actual T(t) data. Sadeghi and Swartzel (1990) further demonstrated that calibration materials (sucrose pH(2.5), blue #2 pH(11.3), and blue#2 pH(9.5)) could be used to evaluate and define thermal homogeneous fluid systems simulating actual conditions (Reynolds number, Nusselt number, shear stress at the wall,.., etc.). The objective of our study was focused on extending the new methodology to thermal systems containing discrete particulates.

MATERIALS & METHODS

IDEALLY, by immobilizing or encapsulating (1-3 mm in diameter) the calibration materials, and placing them within a particle exposed to an unknown thermal treatment, a time-temperature equivalent (t_E,T_E) could be determined. To test the concept, a 20% sucrose solution acidified with sulfuric acid to pH 2.5 and blue #2 buffered (sodium carbonate) solutions at pH 9.5 and 11.3 were encapsulated in aluminum cylindrical modules, 1/4" O.D. of various lengths. Ends of the modules were sealed with silicon sealant, which is heat resistant and stable to 400°C. The constituents were placed into the modules by hypodermic syringes. Although these modules were too large to be incorporated into food particles, they allowed ease in testing methodology. This technique was tested for duplicate samples in a retort operation.

The modules were placed at the center of 303 cans filled with sweet potatoes and water. The cans along with the modules were processed between 110–125°C. After heating, the cans were cooled with tap water. The solutions inside the modules were recovered and diluted to 1ml. Constituent changes occurring in each individual module were assayed following the procedures outlined by Sadeghi (1987).

Ecklund thermocouples were installed into the same can within 1-8 mm of the modules to record time-temperature profiles (Fig. 1). The data were collected on a data logger (Speedomax W Recorder, Leeds & Northrup Co. North Wales, PA.). The time-temperature equivalence for both the thermocouple and calibration material data were determined (Table 1). For the calibration material data (following first order kinetics), t_E and T_E were determined from:

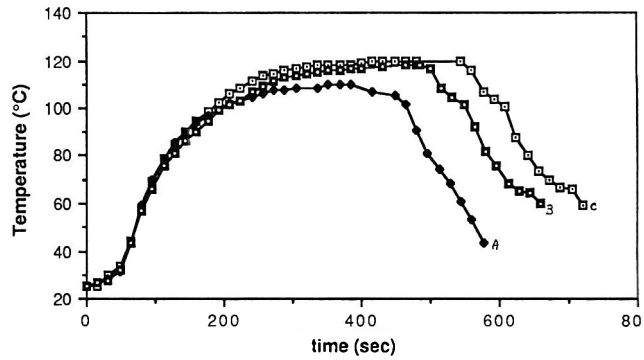

Fig. 1.—Time-temperature profile curves for thermal treatments of canned sweet potatoes.

Table 1—Time-temperature equivalence determined from calibration materials and thermocouples

	Calibration materials	Ecklund thermocouple
(T_E,t_E)	(110.1° C, 3.81 min)	(107.8° C, 3.41 min)
	(114.7° C, 4.30 min)	(116.1° C, 4.45 min)
	(117.1° C, 5.13 min)	(118.4° C, 5.21 min)

$$G_1 = \frac{\ln\left(\frac{C_{1,0}}{C_{1,f}}\right)}{A_1} \qquad (3)$$

and

$$G_2 = \frac{\ln\left(\frac{C_{2,0}}{C_{2,f}}\right)}{A_2} \qquad (4)$$

where indexes 1 and 2 refer to blue #2 (pH 9.5) and sucrose, respectively and C_0 and C_f are the initial and final concentrations. For purposes of examining the methodology, we decided two materials were sufficient based on results by Sadeghi and Swartzel (1990). The equivalent point (t_E,T_E), using (Ea_i, A_i) values of (94.6 kJ/mol, 2.20×10^{10} sec^{-1}) and (74.5 kJ/mol, 1.52×10^{8} sec^{-1}) respectively, for sucrose and blue #2 pH 9.5 (Sadeghi and Swartzel, 1990), was evaluated as:

$$T_E = \frac{Ea_2 - Ea_1}{R \ln\left(\frac{G_1}{G_2}\right)} \qquad (5)$$

$$t_E = \frac{G_1}{\exp\left(\frac{-Ea_1}{RT_E}\right)} \qquad (6)$$

When more than two materials (> 2 Ea values) are used, the LI on WLSLR methods should be incorporated to evaluate t_E and T_E.

RESULTS & DISCUSSION

THE INITIAL and final concentrations of the encapsulated calibration materials were determined after processing in a retort. By utilizing the EPM in reverse and equations 3 through 6, time/temperature equivalents were calculated for each processing condition, averaged, and are shown in Table 1. The thermal curves (Fig. 1) were obtained at various conditions using Ecklund thermocouples. The equivalent point, (t_E,T_E), was determined for each heating condition from the thermocouple collected data, averaged, and shown in Table 1. T_E and t_E determined from the calibration materials for both test runs

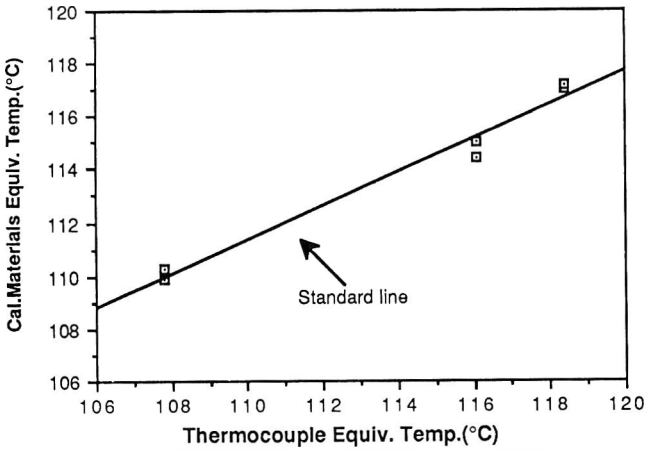

Fig. 2.—Plot of thermocouple equivalent temperatures vs the calibration materials equivalent temperatures.

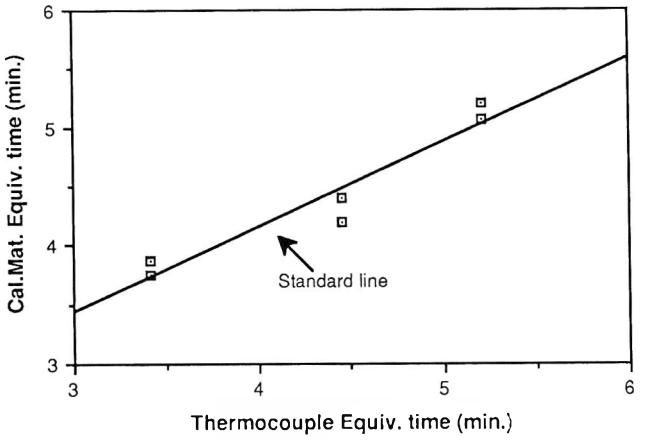

Fig. 3.—Plot of thermocouple equivalent time vs. the calibration materials equivalent time.

Table 2—F_o-values determined from mathematical method (using thermal curves), calibration materials and ecklung thermocouples (using Eq. 7)

	Calibration materials	Ecklund thermocouple	Mathematical method
F_o	0.31 min	0.16 min	0.15 min
	1.01 min	1.44 min	1.34 min
	2.09 min	2.86 min	2.81 min

were separately plotted against the thermocouple T_E and t_E (Fig. 2, 3).

Sadeghi (1987) explained that by utilizing the time-temperature equivalence, the F_o-values can be determined as:

$$F_0 = t_E \, 10^{(T_E - 121°C)/10} \qquad (7)$$

Utilizing Eq. (7) along with time-temperature equivalent points (Table 1), and the traditional mathematical method (Stumbo, 1973) using thermal curves (Fig. 1), F_o-values were determined as shown in Table 2. Care should be exercised in encapsulating or immobilizing such constituents. The problem exists in recovering enough product for analysis. We believe the discrepancies between F_o-values may, in part, be contributed to lack of sufficient product.

Proper selection of calibrating materials is very important. Thus, to avoid extrapolations, calibrating materials should have activation energies covering the range of interest. Concentration changes must be accurately measured to reduce errors in the G_i-values which affect estimated parameters. Error propagation analysis can be used to find the order of magnitude for the frequency factor. For example, proportional errors in C_1 will affect T_E; therefore, using Eq. (3), (4), (5), and (6) the following restriction applies to A_1:

$$A_1 > \frac{2\,T_E R}{|Ea_2 - Ea_1|} \frac{\exp\left(\frac{Ea_1}{R\,T_E}\right)}{t_E} \frac{\frac{\Delta C_1}{C_1}}{\frac{\Delta T_E}{T_E}} \qquad (8)$$

Using $\Delta C_1/C_1 = 0.03$, $\Delta T_E/T_E = 0.005$, $T_E = 117.1$ °C, and $t_E = 5.13$ min, Eq. (8) requires that $A_1 > 2.86\ 10^{10} s^{-1}$ which is close to the experimental value $2.20\ 10^{10} s^{-1}$. Note that Eq. (8) can be used to estimate A_1 for faster heating rates, i.e., for processes having higher T_E's and lower t_E's. Thus, for $T_E = 140$ °C, and $t_E = 30$ s, A_1 must be greater than $6.18\ 10^{10} s^{-1}$.

Thermocouples should have been placed at the center of the modules to record their actual time-temperature profile. However, in our study thermocouples were placed outside within 1 to 8 mm of the centrally located modules. Consequently, the recorded temperatures was slightly higher than those at the module center. This may explain in part why the equivalent temperature for the Ecklund thermocouples were higher than those of the calibration materials, except for the first set (Table 1). In addition, as shown in Table 2, this could also explain similar trends regarding F_0-values.

With this method the residence time distribution effect can be examined. By recovering and analyzing each individual capsule a residence time distribution is established based on concentration changes. This makes possible determination of a (t_E, T_E) for not only the particle with the mean velocity, but also for the particle with either the greatest velocity or the least velocity. In addition the procedure allows each individual capsule to be considered simulating actual conditions. This procedure is independent of particle size or shape since calibration materials are placed within the particle.

Miniaturization plays an important role for further developments of these concepts. Consequently, the size of the modules must be as small as technically possible in order to use this technique with different particle sizes. Clearly, more experimental evidence is needed, especially or continuous flow systems at high temperatures.

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—*Continued on page 1739*

Radiolytic Degradation of Sorbic Acid in Isolated Systems

B.R. THAKUR, I.R. TREHAN, and S.S. ARYA

ABSTRACT

Effect of Co^{60} γ-irradiation on stability of sorbic acid (SA) in solutions, dough and chapaties has been investigated. SA was highly susceptible to radiolytic degradation in aqueous systems. Rate of degradation decreased with rise in pH. Sugars, hydrocolloids except pectin, citric acid, lactic acid, malic acid, arginine and threonine, catalysed degradation while oxalic acid, maleic acid, Cu^{2+}, nitrite, nitrate and phthalate had protective effects. SA was more stable in alcohols and vegetable oils than in aqueous solutions. In wheat flour radiolytic degradation of SA was less at lower moisture. Relatively SA was more stable in chapaties than in dough. Gelatinization and addition of oil in dough reduced degradation of SA.

INTRODUCTION

IRRADIATION for food preservation has been widely investigated. Foods irradiated up to 10 KGy are now generally accepted as safe and do not pose any toxic hazard (Urbain, 1978). However, this dosage is insufficient for sterilization of foods and some workers are trying irradiation plus added antimicrobial compounds to impart desired shelf stability (Eisenberg et al., 1969; Iordanov, 1972; Licciardello et al., 1984). Ishizaki et al. (1972) reported sorbic acid in aqueous solution undergoes degradation on irradiation and the radiolytic degradation product(s) had higher antimicrobial activity than the parent compound. In our laboratory combined use of sorbic acid (SA) and γ-irradiation is being studied for preservation of chapaties, as irradiation alone, up to 10 KGy has not been effective in providing 6 months shelf life. During the course of our studies we observed that SA underwent considerable degradation during irradiation of chapaties and both dosage and dough ingredients influenced radiolytic degradation of SA. (unpublished data).

During processing of preserved chapaties several additives such as salt, citric acid, sugar, vanaspati, flavoring compounds and hydorcolloids are employed to retain soft texture and fresh flavor during storage. These, as well as type of flour, could influence rate of radiolytic degradation of SA. The purpose of our study was to investigate the radiolytic degradation of SA in isolated systems.

MATERIALS & METHODS

Materials

Chemicals: SA, glycine, lysine, leucine, serine, threonine, methionine, cellobiose, raffinose, galactose, sorbose and lactose were from E. Merck, Germany. Glucose, sucrose, maltose, arginine, citric, maleic, malic and oxalic acids were from BDH Chemicals Ltd. Poole, England while β-carotene was from Sigma Chemicals, USA. All reagents were analytical reagent grade and used without further purification. Ethanol was refluxed with zinc dust and 0.25M NaOH for 2 hr and distilled in all-glass apparatus before use.

Wheat flour (atta) and other flours: Sharbati wheat (*Triticum aestivum*) was ground in a commercial mill and sieved through 30 mesh to obtain 90% extraction flour. Bengalgram (*Cicer arietinum*), black gram (*Phaseolus mungo*), redgram (*Cajanus cajan*) dehusked pulses, polished rice (*Oryza sativa*) and sorghum (*Sorghum vulgare*) were powdered in an ultracentrifugal mill (Retsch Type Z.M.I. W. Germany) in the laboratory and sieved through 30 mesh.

Vegetable oils: Refined, bleached and deodorized oils of sunflower, safflower, peanut, coconut and vanaspati (hydrogenated vegetable oil, I.V. 70-75) were procured from the local market.

Chapati: Dough was prepared by mixing wheat flour (300g), water (180 mL) and SA (0.9g) in a farinograph mixer with and without salt and vegetable oils. Dough was divided into 20g pieces shaped by hand and rolled into circular discs of 7.5 cm and cooked on a hot plate at 220°C exactly 2 min as described (Arya et al., 1977), but without applying vegetable oil at time of cooking. Two chapaties were packed in PFP laminate [paper (45 GSM)-Al foil (20 μ)-polyethylene (37.5 μ)] pouches and sealed for irradiation.

Irradiation: Two mL aliquots of SA in ethanol (100% w/v) were diluted to 100 mL with water. Requisite amounts of sugars, amino acids, hydrocolloics, salts and other reagents were added before making up the volume. Aliquots (10 mL) of the SA solution were irradiated in stoppered glass tubes in a Cobalt 60 irradiator (Gamma Chamber 4000A-Trombay, Bombay, BARC) at dosage levels ranging from 2.5 to 20 KGy at 0.5376 KGy/hr at room temperature (18–32°C). Chapaties and doughs from various ingredients were irradiated after packing in PFP laminate pouches in 40g lots.

To study the effect of moisture on SA degradation, wheat flour (atta) was dried 4 hr in an oven at 100°C. Dried material (300g) was added to 300 mL SA solution (0.20% w/v, chloroform) in a 1-L round bottom flask and thoroughly mixed to obtain a homogeneous slurry. The solvent was evaporated under vacuum using a rotary evaporator. SA impregnated wheat flour (100 g) was mixed with known amounts of water and thoroughly mixed in a glass mortar to obtain homogeneous samples which were packed in PFP laminate pouches, sealed and irradiated. All irradiation experiments were conducted in triplicate.

Analysis: In solid, semi-solid and oil systems, SA was determined by the method described previously (Vidyasagar and Arya, 1984). In aqueous solutions, the SA was determined by measuring absorbance at 258 nm after appropriate dilutions. Free fatty acid and peroxide values were determined by standard AOCS (1973) methods. Moisture in atta, dough and chapaties was determined by heating 1 hr in an air oven at 130°C. All analyses were in duplicate and results are means of a minimum of four values. Mean and standard deviation were calculated by standard statistical methods and significance of differences in the various treatments and control were tested by Student's 't' test (Ostle, 1965).

RESULTS & DISCUSSION

SA EXHIBITS a strong absorption maximum at 258 nm due to the 2, 4-conjugated diene in its structure. Irradiation of aqueous solution of sorbic acid resulted in a decrease in absorbance at 258 nm with simultaneous appearance of another absorption maximum around 195 nm (Fig. 1). Evaporation of water from irradiated SA solution under vacuum gave a viscous product which was repeatedly extracted with chloroform until free of unreacted sorbic acid. The purified product, dissolved in methanol, had an absorption maximum at 210 nm (Fig. 1). Autoxidation of SA in aqueous solutions also caused decrease in absorbance maximum at 258 nm with a simultaneous increase in concentration of carbonyl compounds (Arya, 1980). However, irradiation of SA did not result in formation of carbonyl compounds in appreciable amounts. Neither did browning intensity increase. Browning resulted from polymerization of unsaturated carbonyls in autoxidizing SA solution (Arya and Thakur, 1988).

The degradation of SA in aqueous solution increased linearly

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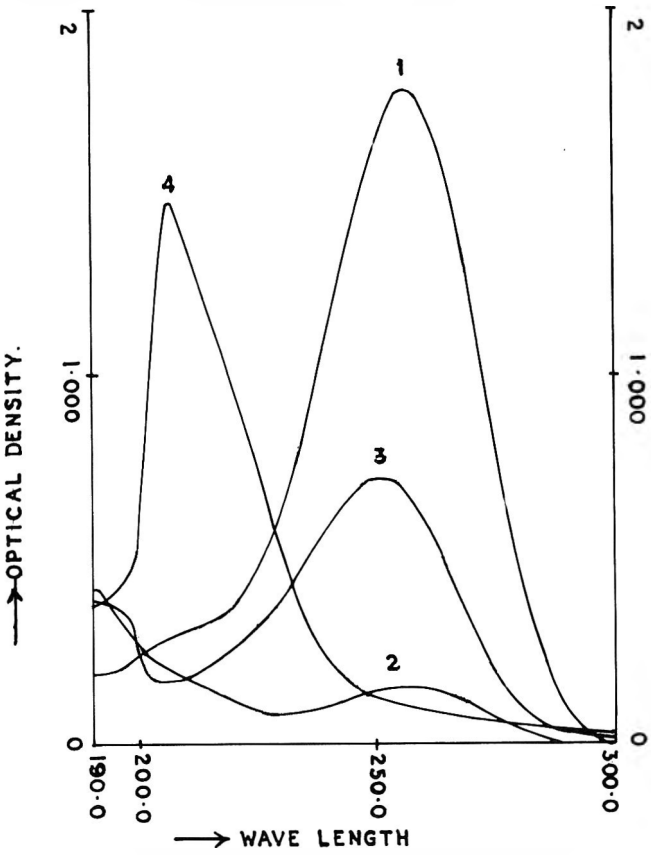

Fig. 1—Effect of irradiation on absorption spectrum of sorbic acid (SA). 1 & 2, Aqueous SA solution before and after irradiation (8 x 10^{-4} g/100 mL); 3, SA in methanol (3 x 10^{-4} g/100 mL); 4, Radiolytic degradation product of SA in methanol, (1.5 x 10^{-2} g/100 mL).

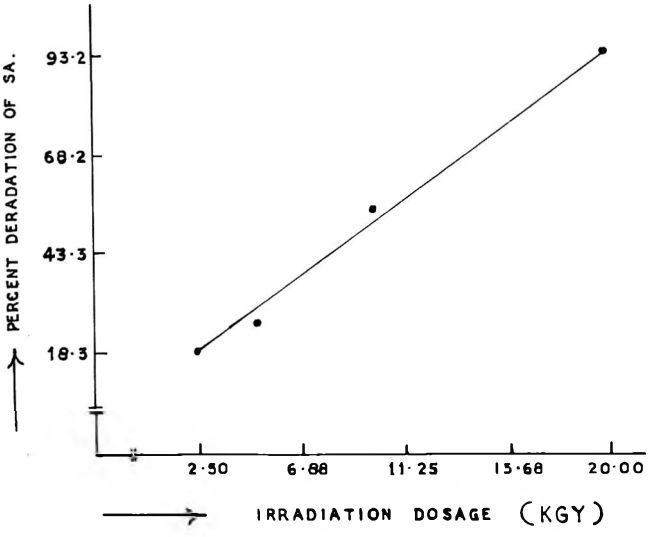

Fig. 2—Effect of irradiation dosage on the degradation of sorbic acid in aqueous solutions.

with irradiation dose from 2.5 to 20 KGy (Fig. 2). All values could be fitted in the regression equation (Y = 9.19 + 4.28 X), correlation coefficient 0.986 with 95% confidence limits. Also, SA in undissociated form was more susceptible to irradiation degradation as indicated by its higher rate of degradation at lower pH (Fig. 3). The relationship of percent SA degraded at different pHs could be fitted to a linear regression equation (Y = 65.14 − 3.79 X) with correlation coefficient of 0.957. Higher susceptibility of fatty acids to radiolytic deg-

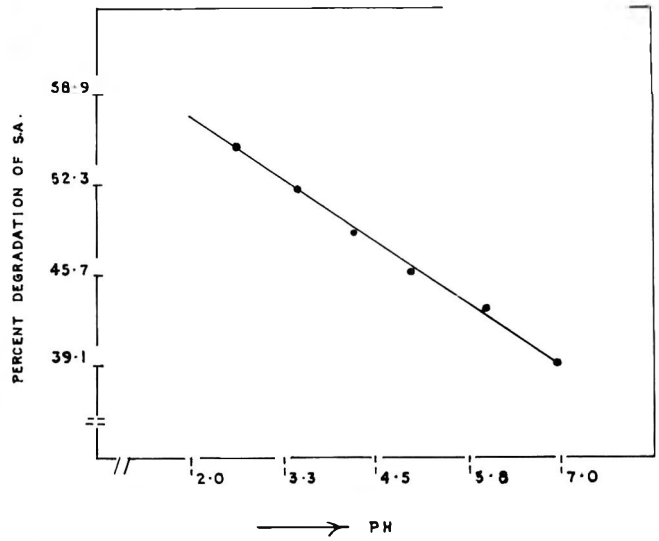

Fig. 3—Effect of pH on the radiolytic degradation of sorbic acid at a dosage of 10 KGy.

Table 1—Effect of solvents on radiolytic degradation of sorbic acid (0.2% SA, w/v)

Solvent	Percentage degradation
SA + water (control)	48.2 ± 2.2
SA + Methanol	21.7 ± 1.0
SA + Ethanol	26.0 ± 1.0
SA + Butanol	28.7 ± 1.0
SA + Chloroform	51.0 ± 0.2
SA + Ethanol (10%)	49.6 ± 1.7*
SA + Ethanol (20%)	49.6 ± 1.9*
SA + Ethanol (40%)	47.6 ± 2.1*
SA + Ethanol (60%)	41.8 ± 2.5
SA + Ethanol (100%)	26.0 ± 1.0

* Not significantly different from control at 95% confidence.

radation at lower pH has been reported (Simic, 1983) due to increased reactivity of undissociated carboxyl groups toward hydrated electrons. Ionized carboxyl groups are relatively unreactive towards hydrated electrons. Higher susceptibility of SA to radiolytic degradation at lower pH may also be due to the higher yields of primary radicals formed from water in acidic solutions. Spinks and Woods (1964) discussed the role of pH on yields of free radicals from radiolysis of water. Radical yields and G-H_2O have been reported significantly higher in acid than in neutral solution. In autoxidative degradation also, SA was found to degrade faster at lower pH (Arya, 1980).

The nature of the solvent also significantly influenced radiation degradation of SA (Table 1). As compared to water, degradation was considerably less in alcohols (ethanol, methanol, isopropanol and butanol). In ethanol-water mixtures having ethanol below 40% SA degradation was not significantly ($p \leq 0.95$) different from water. But in mixtures having ethanol above 60%, SA degradation was significantly ($p \geq 0.95$) less than in water. Among different organic solvents, degradation was highest in chloroform and lowest in isopropanol. Rate of degradation was also different in four alcohols studied, being highest in n-butanol and lowest in isopropanol. Differences in rates of degradation in various solvents were significant ($p \geq 0.999$, $t > 7.235$, $n = 6$) at 99.9% confidence.

Effect of solvents on radiolytic degradation of riboflavin has also been studied by Kishore et al. (1978). These workers also observed reduced radiolytic degradation of riboflavin in methanol and ethanol in comparison to water. In their study, isopropanol and t-butanol did not exert significant protective effects, but in our study isopropanol had maximum protective action in the radiolytic degradation of SA.

In dilute solutions the degradation of solute molecules is

Table 2—Effect of various sugars (0.2M), amino acids (0.2M) and hydrocolloids (2%, w/v), on radiolytic degradation of sorbic acid (0.2% SA, w/v)

Added component	Percentage degradation[a]
SA + Control	48 ± 2.2
SA + Glucose	56 ± 2.1
SA + Fructose	54 ± 2.9
SA + Maltose	54 ± 2.6
SA + Galactose	55 ± 2.8
SA + Sorbose	55 ± 3.2
SA + Sucrose	56 ± 3.5
SA + Sucrose (10%)	62 ± 0.2
SA + Sucrose (20%)	63 ± 0.4
SA + Sucrose (40%)	69 ± 0.3
SA + Sucrose (60%)	74 ± 0.7
SA + Lactose	55 ± 2.6
SA + Cellobiose	55 ± 1.9
SA + Raffinose	56 ± 2.8
SA + Mellibiose	53 ± 1.1
SA + Gelatin	56 ± 0.9
SA + Locust bean	65 ± 0.9
SA + Carboxymethyl cellulose	59 ± 1.8
SA + Xanthin	69 ± 1.0
SA + Pectin	41 ± 1.4
SA + Arginine	56 ± 0.5
SA + Threonine	53 ± 1.8

[a] All treatments were significantly different from control at 95% confidence limits.

Table 3—Effect of carboxylic acids (0.2 M) and salts (0.2 M) on the radiolytic degradation of sorbic acid (0.2% SA, w/v)

Added Salts	Percentage degradation[a]
SA + Control (3.30)[b]	48.2 ± 2.2
SA + Sodium chloride (3.50)	41.7 ± 1.1
SA + Sodium hydrogen phosphate (8.12)	35.9 ± 1.2
SA + Sodium dihydrogen phosphate (4.31)	39.1 ± 1.3
SA + Sodium carbonate (11.28)	35.8 ± 1.3
SA + Sodium bicarbonate (8.93)	34.9 ± 1.8
SA + Sodium nitrite (5.52)	3.0 ± 1.2
SA + Sodium nitrate (3.46)	19.5 ± 1.6
SA + Sodium citrate (6.86)	30.8 ± 2.3
SA + Sodium acetate (5.83)	33.7 ± 2.7
SA + Sodium sulphate (4.03)	41.2 ± 1.1
SA + Sodium thiosulphate (4.39)	22.8 ± 0.7
SA + Potassium bicarbonate (8.15)	32.9 ± 2.0
SA + Potassium nitrate (3.58)	17.8 ± 1.5
SA + Potassium chloride (3.50)	34.0 ± 1.1
SA + Potassium oxalate (5.45)	30.2 ± 2.5
SA + Potassium sulphate (4.04)	39.5 ± 1.3
SA + Potassium hydrogen phthalate (4.45)	23.2 ± 1.6
SA + Citric acid (1.70)	56.3 ± 1.5
SA + Lactic acid (1.82)	57.3 ± 0.3
SA + Malic acid (1.93)	59.4 ± 3.2
SA + Maleic acid (1.63)	35.9 ± 3.0
SA + Oxalic acid (1.52)	44.2 ± 1.5

[a] All treatments were significantly different from control at 99.9% confidence limits.
[b] Values in parenthesis indicate the pH of the system.

mainly brought about by their reaction with free radicals formed by dissociation of solvent molecules. The effect of γ-radiation on alcohols has been discussed by Spinks and Woods (1964). In alcohols, free radicals are formed by loss of an α-hydrogen atom as a result of scission of the carbon-hydrogen bond. The various radicals expected from methanol, ethanol, isopropanol and n-butanol include $.CH_2OH$, $CH_3\dot{C}HOH$, $(CH_3)_2\ \dot{C}HOH$ and $C_3H_7\ \dot{C}HOH$ respectively. Normally the rates of reactions of free radicals are governed by viscosity and dielectric constant of the medium. However, this was not the case in radiolytic degradation of SA in alcohols and water. Very low degradation of SA in isorpropanol and methanol suggested the high susceptibility of $(CH_3)_2\ \dot{C}HOH$ and $.CH_2OH$ radicals to further rearrangements probably forming carbonyls and hydrogen ions.

$$\cdot CH_2OH \rightarrow HCHO + H^+$$

However, specific evidence for occurrence of these reactions remains to be established.

Effects of sugars, hydrocolloids and amino acids on radiolytic degradation of SA are shown in Table 2. At 0.2M concentration all sugars had catalytic effects in the radiolytic degradation of sorbic acid. The losses in SA at 10 KGy dose ranged from 53 to 56% in presence of sugars as compared to 48% in control samples. Differences in control and sugar treated samples were significant at $p \geq 0.975$. Effect of sugars was also concentration dependent and when the levels of sucrose increased from 10% to 60%, SA degradation also increased from 62 to 74% at 10 KGy irradiation dose. Differences in rates of SA degradation at different sucrose concentrations were significant at $p \geq 0.999$ ($t \geq 6.239$, $n = 6$). The catalytic action of sugars in radiolytic degradation of SA was unexpected because previously sugars had exhibited protective action in radiolytic degradation of proteins (Diehl et al. 1978), and vitamins (Kishore et al., 1978; Bhushan and Kumta, 1977). Sugars have been reported to react with hydroxyl radicals and thereby act as competitive inhibitors (Diehl et al. 1978). However, this was not observed in radiolytic degradation of SA in aqueous solutions.

All hydrocolloids (gelatin, locust bean gum, carboxy methylcellulose and xanthin) except pectin also accelerated the rate of radiolytic degradation of SA (Table 2). Losses in SA in the presence of hydrocolloids (2%, w/v) ranged from 56% to 69% as compared to 41% in presence of pectin and 48% loss in the contol at 10 KGy dose.

The effect of amino acids and carboxylic acids has been rather selective. While glycine, leucine, lysine and serine did not exert significant catalytic effects at 95% confidence ($t < 1.935$, $n = 6$), arginine, threonine, citric, malic and lactic acids significantly ($p \geq 0.975$, $t \geq 3.447$, $n = 6$) accelerated rate of degradation (Table 2, 3). On the other hand, oxalic acid and maleic acid had a significant ($p \geq 0.975$, $t \geq 3.003$, $n = 6$) protective action, the losses being 35% to 44% as compared to 48% loss in controls (Table 3). Degradation of SA in the presence of potassium oxalate (0.2 M) was less (30.2%) than that of oxalic acid (44.2%). This was expected because of higher pH of the potassium oxalate system (pH 5.45) than that of the oxalic acid system (pH 1.52).

In autoxidizing SA almost all salts except sodium and potassium chlorides had pro-oxidant effects (Arya, 1980). But in radiolytic degradation, all salts studied (Table 3) exhibited significant ($p > 0.999$ $t \geq 5.288$, $n = 6$) protective effects. Losses in SA ranged from 3% to 42% in presence of various salts (0.2 M) compared to 48.2% in the control. Relatively, radiolytic degradation was least in presence of nitrate (3%), nitrate (19.5%) and potassium hydrogen phthalate (23.2%) and highest in presence of chlorides (42%). Both nitrite and nitrate ions are excellent scavengers of hydrated electrons (Simic, 1983). Nitrite is also highly reactive towards hydroxyl radicals and therefore lesser degradation of SA in their presence is in conformity with published information. Aromatic compounds are also highly reactive to hydroxyl radicals (Simic, 1983) and therefore, phthalate molecules were expected to compete with SA in reaction with hydroxyl radicals and thereby act as competetive inhibitors. Aromatic compounds were previously reported (Spinks and Woods, 1964) to have protective action in radiolytic degradation of aliphatic compounds. Chloride, sulphate and phosphate radicals were reported (Simic, 1983) totally unreactive towards hydroxyl radicals and hydrated electrons and accordingly salts of sodium and potassium with those ions did not have much protective action. Slight protective action in their presence may be due to increase in pH.

The role of transition metal ions in autoxidative reactions in foods is quite complex. While transition metal ions accelerate autoxidation of fats and oils, these had a strong inhibitory effect in the autoxidative degradation of SA in aqueous solutions. In radiolytic degradation too, transition metal ions were expected to participate through reaction with free radicals and

Table 4—Changes in peroxide value (meq of O_2/kg fat), free fatty acid (% oleic acid) and thiobarbituric acid (mg malonaldehyde/kg fat) values of oils on irradiation (10 KGy)

Oil type	Peroxide value		Free fatty acid		Thiobarbituric acid*	
	Control	Irradiated	Control	Irradiated	Control	Irradiated
Sunflower	12.0 ± 2.5	12.5 ± 1.8	0.23 ± 0.01	0.24 ± 0.03	0.15 ± 0.02	0.21 ± 0.03
Sunflower + SA	13.4 ± 2.2	13.5 ± 2.4	0.74 ± 0.06	0.75 ± 0.03	0.23 ± 0.03	0.42 ± 0.04
Coconut	3.6 ± 1.0	3.8 ± 0.8	1.12 ± 0.10	1.16 ± 0.05	0.10 ± 0.01	0.46 ± 0.04
Coconut + SA	4.6 ± 1.2	3.9 ± 1.8	1.61 ± 0.30	1.62 ± 0.1	0.21 ± 0.02	0.61 ± 0.05
Vanaspati	4.8 ± 1.0	5.0 ± 0.7	0.19 ± 0.02	0.18 ± 0.01	0.09 ± 0.01	0.20 ± 0.03
Vansapati + SA	4.7 ± 1.0	5.0 ± 0.6	0.63 ± 0.02	0.69 ± 0.05	0.45 ± 0.05	1.04 ± 0.07

* TBA values of irradiated samples significantly different from corresponding unirradiated controls at 99.9% confidence.

hydrated electrons. Metal ions in their higher redox states are generally reactive with hydrated electrons while hydroxyl radicals tend to react with them in lower redox state.

In our study the effect of Cu^{2+}, Fe^{2+}, Co^{2+} and Ni^{2+} on radiolytic degradation of SA was also investigated. The percentage degradation of SA in presence of 100 ppm of Cu^{2+}, Fe^{2+}, Co^{2+} and Ni^{2+} was found to be 28.0, 52.1, 48.0, and 47.5%, respectively, as compared to 48.2% in controls indicating a significant protective effect of Cu^{2+} only. Effect of other transition metal ions was not significant at 95% confidence.

Compared to aqueous solutions, radiolytic degradation of SA was negligible in fats and oils. There was no degradation of SA when irradiated in sunflower and safflower oils while degradation in vanaspati (hydrogenated vegetable oil), coconut oil and groundnut oil was 15.7%, 17.8% and 4.5%, respectively. This suggested the beneficial effects of polyunsaturated fatty acids in protecting SA. Differences in rates of degradation of SA in coconut oil and vanaspati were not significant at 95% confidence ($t \leq 1.053$, $n = 6$). Between peanut oil and coconut oil, vanaspati or sunflower oil, differences were significant ($p \geq 0.999$, $t \geq 17.710$, $n = 6$). Probably polyunsaturated fatty acids reacted with free radicals and thus protected SA from free radical attack. Except slight increase in TBA value of vegetable oils, the changes in free fatty acids and peroxide value of oils were not significant (Table 4).

The effect of moisture and processing factors on degradation of SA in atta (flour), dough and chapati is given in Table 5. The radiolytic degradation of SA in these systems was dependent on moisture. In dry atta, only 23% of SA was degraded as compared to more than 64% degradation at 10% moisture (Table 5). In wheat flour dough also about 63% of added SA was degraded as compared to 46% to 49% degradation in chapaties. Among the various cereals and pulse flours, degradation was highest in rice flour dough and lowest in bengalgram flour dough. Differences in rates of SA degradation among doughs from wheat flour, rice flour, sorghum flour and redgram flour were significant at $p \geq 0.999$. But between blackgram flour dough and bengalgram flour dough, differences were significant at $p \geq 0.975$. Between wheat flour dough and bengalgram flour dough, differences were not significant at $p = 0.95$. Heating doughs in boiling water 5-10 minutes also considerably reduced radiolytic degradation of SA suggesting protective effect of starch gelatinization on radiolytic degradation of SA. Stabilizing effect of gelatinization and fat in radiolytic degradation of SA was significant at $p \geq 0.999$. Also, stabilizing effects due to gelatinization and fat were additive in wheat and rice flour doughs. Inclusion of fat in dough and chapaties reduced SA degradation during subsequent irradiation. This is in conformity with the very slow rate of degradation of SA in fats/oils. Inclusion of sucrose and sodium chloride in wheat flour dough, however, did not significantly ($p \leq 0.95$) influence rate of radiolytic degradation of SA in dough or in chapaties.

In moist systems the primary action of irradiation is the radiolysis of water forming hydrated electrons, hydroxyl radicals and hydrogen atoms. The primary products are transient in nature and disappear either in reaction with each other or with SA and/or other food constituents. These reactions are

Table 5—Effect of different food materials, gelatinization, fat and moisture on radiolytic degradation of sorbic acid (0.2% SA, w/v)

Food material	Percentage degradation
Wheat flour (0.1% moisture)	23 ± 3.5
Wheat flour (10% moisture)	64 ± 2.1
Wheat flour dough	63 ± 1.5
Gelatinized wheat flour dough	40 ± 1.6[b]
Wheat flour dough with fat (20%)	56 ± 1.8[c]
Gelatinized wheat flour dough with fat (20%)	17 ± 0.5
Chapati	47 ± 1.5[a]
Rice flour dough	94 ± 0.5
Gelatinized rice flour dough	59 ± 0.4[b]
Rice flour dough with fat (20%)	62 ± 2.0[c]
Gelatinized rice flour dough with fat (20%)	25 ± 2.0
Sorghum flour dough	77 ± 1.5[a]
Gelatinized sorghum flour dough	42 ± 2.0[b]
Bengalgram flour dough	61 ± 2.0
Gelatinized bengalgram flour dough	29 ± 2.6[b]
Blackgram flour dough	64 ± 0.7
Gelatinized blackgram flour dough	31 ± 1.0[b]
Redgram flour dough	68 ± 1.5[a]

[a] Significantly different from wheat flour dough at 99.95% confidence limits.
[b] Significantly different from the corresponding ungelatinized flour doughs at 99.95% confidence limits.
[c] Significantly different from the corresponding dough without fat at 99.95% confidence limits.

very fast, of the order of $10^{10} - 10^{11}$ $M^{-1}S^{-1}$ and reaction rates are controlled by diffusivity of the primary reaction products (Simic, 1983). Among primary radiolytic products of water, hydroxyl radicals are most reactive towards unsaturated compounds (Simic, 1983) especially those with conjugated polyene chain. Hydrated electrons and hydrogen atoms can also react with conjugated polyene chains at rates forming free radicals by addition reactions. High susceptibility of SA to irradiation degradation may therefore be attributed to conjugated polyene structure. The rate of degradation of SA in various systems will be governed by diffusivity of hydroxyl radicals, hydrated electrons and hydrogen atoms and also by the presence of other solutes which may compete with SA in reaction with radiolytic products of water. Since heating of dough increases its viscosity from starch gelatinization, protein denaturation and increase of bound water, lesser degradation of SA in chapaties and gelatinized dough was in conformity.

With vegetable oils, primary radicals formed are much bulkier (Nawar, 1978) and therefore these will be lesser mobile than hydroxyl radicals. Consequently, chances of these radicals interacting with SA would be rather low and therefore SA was expected to remain stable in fats and oils. In fact degradation of SA in fats and oils may have been a result of direct interaction with radiation energy rather than reaction with other free radicals formed as a result of radiolysis of oils. With highly unsaturated vegetable oils, the major portion of energy is absorbed by polyunsaturated fatty acids because of thier very high concentration. SA in these oils therefore remained stable as observed in our study. In saturated oils larger proportions of energy are absorbed by polyunsaturated SA molecules and therefore SA was expected to be more degraded.

From the foregoing discussion it was evident that degradation of SA in chapaties, doughs and solutions was governed by several factors like moisture, extent of gelatinization, nature

—Continued on page 1710

Velocity Distributions of Food Particle Suspensions In Holding Tube Flow: Distribution Characteristics and Fastest-Particle Velocities

BHASKAR DUTTA and SUDHIR K. SASTRY

ABSTRACT

Velocity distributions of model food particles were investigated by videotaping particles suspended in sodium carboxymethylcellulose (CMC) solutions during passage through a transparent holding tube similar in dimension to that of commercial aseptic processing systems. The distributions could be well described by log-normal models. Fastest particle velocities were below theoretical centerline velocities for Newtonian fluids but were above theoretical values for the fluids used. Results indicated that for particle concentration levels used in these studies, channeling and particle-fluid interaction effects may be significant.

INTRODUCTION

A CRITICAL CONSIDERATION in design of safe continuous thermal processes for liquid-particle mixtures is the fastest moving particle. Since this particle possesses the lowest residence time under thermally microbe-lethal conditions, holding tubes must be sized to assure it receives the desired thermal treatment. Although a few studies have been conducted on residence time distributions in swept-surface heat exchangers (Taeymans et al., 1985; Defrise and Taeymans, 1988), studies on holding tube flows have been extremely limited (Nesaratnam and Gaze, 1987; Berry, 1989).

The present studies are part of an ongoing project to characterize velocity distributions of particle suspensions during holding tube flows. Studies on modeling of particle flow and factors influencing means and standard deviations of particle velocities were covered in another report (Dutta and Sastry, 1990). In both the former and this study, the emphasis was on velocity rather than residence time distributions. The latter concept is more useful when flows cannot be easily observed, and information on residence time distributions should be easily obtainable from velocity distribution data. This study had the following objectives: (1) determination of the characteristics of the observed distributions; (2) characterization of fastest-particle velocities as influenced by process variables; and (3) compilation of qualitative observations of particle behavior in tube flow.

MATERIALS AND METHODS

THE EXPERIMENTAL SETUP and materials were described by Dutta and Sastry (1990), consisting of a transparent holding tube through which model particles in a liquid carrier were pumped at room temperature. Particles were spherical with diameter 0.95 cm, and made of polystyrene of representative density (1044.5 kg/m^3). Carriers were solutions of sodium carboxymethylcellulose (CMC). Particle motion in the first leg of the tube was recorded on videotape and viewed for analysis. The levels of experimental variables were the same as in the previous studies; being conducted at flow rates corresponding to three pump speeds (100, 120 and 140 rpm), three levels of carrier viscosity (varied by CMC concentration; 0.2, 0.5 and 0.8%), and four levels of particle concentration (0.2036, 0.4072, 0.6108 and 0.8145% by volume). The principal difference in the study reported here, was in the information sought and the nature of the observations. In the current studies, particle velocity distributions were determined by recording velocities and observing behavior of individual particles in an attempt to gain insight into particle-particle and particle-wall interactions. Particle behavior was noted in detail, and interaction effects were recorded. Data were collected for 100 particles for each combination of variables. At the highest concentrations, when visual particle distinguishability was difficult, data were collected with a set of fluorescent tracer particles, while videotaping in a darkened environment.

For each test particle velocities were normalized by dividing them by the mean fluid velocity. Data on normalized velocities (V_n) were analyzed to determine distribution models using the likelihood ratio test (Lawless, 1982). Models tested included the Weibull, Gaussian and log normal distributions. Distribution parameters were determined for best fitting model(s).

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RESULTS & DISCUSSION

Distribution characteristics

Histograms of velocity distribution are presented in Fig. 1 through 9, in groups of four (one per particle concentration level) at each CMC concentration-pump speed combination. The lowest viscosity-flow rate combination is shown in Fig. 1. At the lowest particle concentration (Fig. 1a), particles stayed mainly at the bottom of the tube, accounting for the low values of normalized velocity for this experiment. Particles were spaced apart by distances of several centimeters in some cases. Particle-particle interactions did not involve many collisions, but occasional "cluster" or "necklace" formation as observed by Segré (1965). Sometimes, two or three particles would cluster and move closely together at the same velocity throughout the tube length. Occasionally, a "leapfrog" motion (one particle moving over and in front of another) would be observed, as described later under particle-particle interactions. Finally, some particles would move slightly faster as a result of being lifted slightly off the tube bottom.

As the particle concentration was increased to 0.4072%, the bottom of the tube had more particles, and several particles were observed to be slightly lifted off the tube bottom and unable to settle completely because of the consistent presence of other particles at the tube bottom. Further discussion on these observations is presented below "Particle-Particle Interactions". Other particles moved by the "leapfrog" motion. The net result was a group of particles that moved (at various velocities) faster than the bottom-rolling majority. The result was a small number of particles that moved considerably faster than the mean fluid velocity, as shown in Fig. 1(b). As particle concentrations increased further (Fig. 1c and 1d), a larger proportion of particles were lifted slightly off the bottom surface, resulting in curve peaks at higher values of V_n.

As the flow rate (pump speed) increased, (Fig. 2 and 3), increasing numbers of particles were lifted off the bottom surface, causing distribution curves to shift to the right. This effect was reflected in positive correlations reported between mean normalized velocity (V_{nm}) and particle Froude number (Fr_p) by Dutta and Sastry (1990). Additionally, the tendency towards cluster formation in bottom layers decreased markedly with increasing flow velocity. At the highest pump speed (140 rpm) very few particles possessed V_n values less than 1. Particle concentration effects did not follow well-defined trends

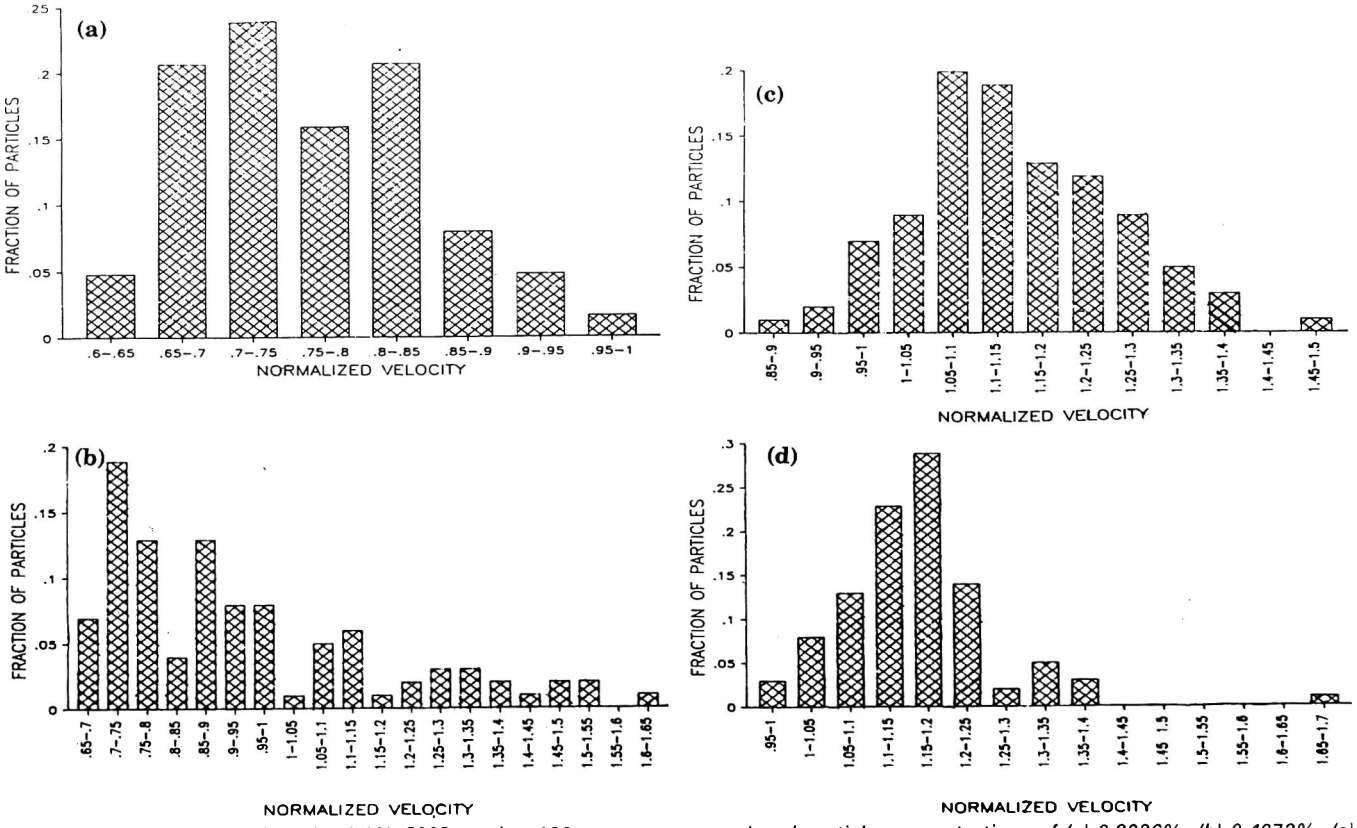

Fig. 1—Velocity distributions for 0.2% CMC carrier, 100 rpm pump speed and particle concentrations of (a) 0.2036%, (b) 0.4072%, (c) 0.6108%, and (d) 0.8145%.

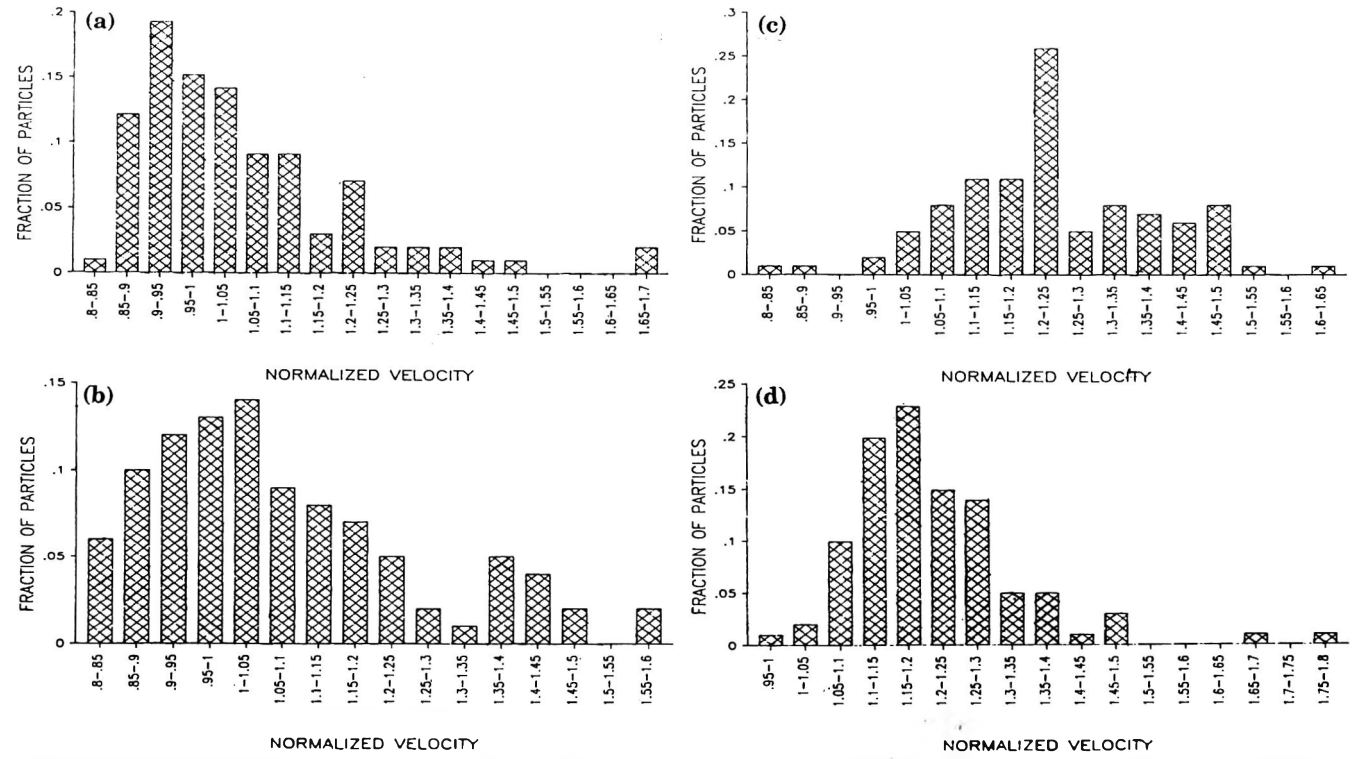

Fig. 2—Velocity distributions for 0.2% CMC carrier, 120 rpm pump speed and particle concentrations of (a) 0.2036%, (b) 0.4072%, (c) 0.6108%, and (d) 0.8145%.

at 0.2% CMC concentration, partly because the relatively low carrier viscosity resulted in relatively free particle motion. Thus, complex particle-particle interactions were observed, resulting in complex particle trajectories. The maximum value of generalized Reynolds number (Re_g) in these studies was below 1200, eliminating the possibility of pure fluid turbulence in the carrier. The effect of particle-induced disturbances on the onset of turbulence has not been definitively quantified.

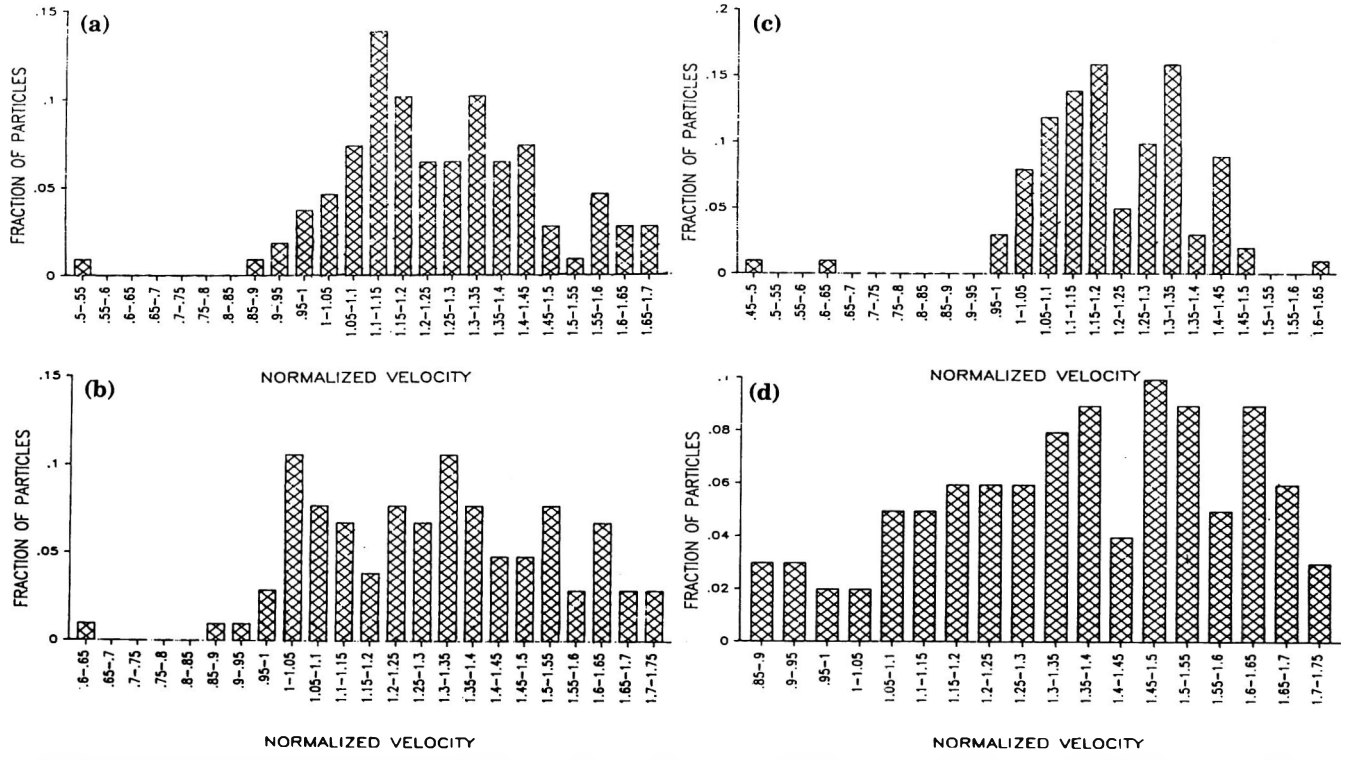

Fig. 3—Velocity distributions for 0.2% CMC carrier, 140 rpm pump speed and particle concentrations of (a) 0.2036%, (b) 0.4072%, (c) 0.6108%, and (d) 0.8145%.

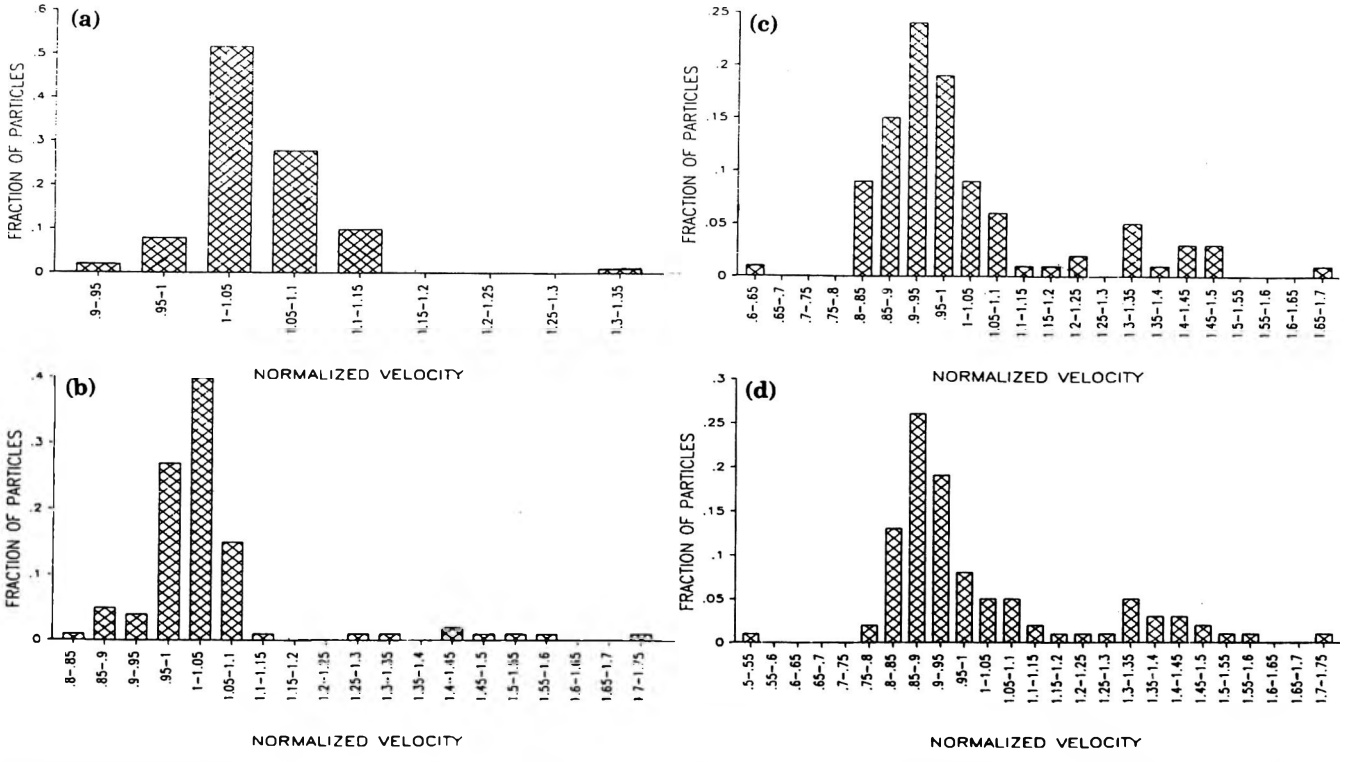

Fig. 4—Velocity distributions for 0.5% CMC carrier, 100 rpm pump speed and particle concentrations of (a) 0.2036%, (b) 0.4072%, (c) 0.6108%, and (d) 0.8145%.

At a higher fluid viscosity (0.5% CMC, Fig. 4-6), the occurrence of V_n below one became infrequent, and the vast majority of particles moved at higher velocities. The influence of particle concentration became well defined at this viscosity level, because particle motion was more restricted, and tended to occur more along streamlines rather than the complex motion reported for 0.2% CMC. Most particles at this viscosity were lifted slightly off the surface, but were below the tube axis. As particle concentration increased, some particles moved above (and at various higher velocities than) this layer; thus resulting in the wide distribution of small numbers of particles at higher velocities observed in the graphic distribution profile as a long "tail". An interesting point we noted was that even at the lower particle concentrations (Fig. 4a and 5a), a few

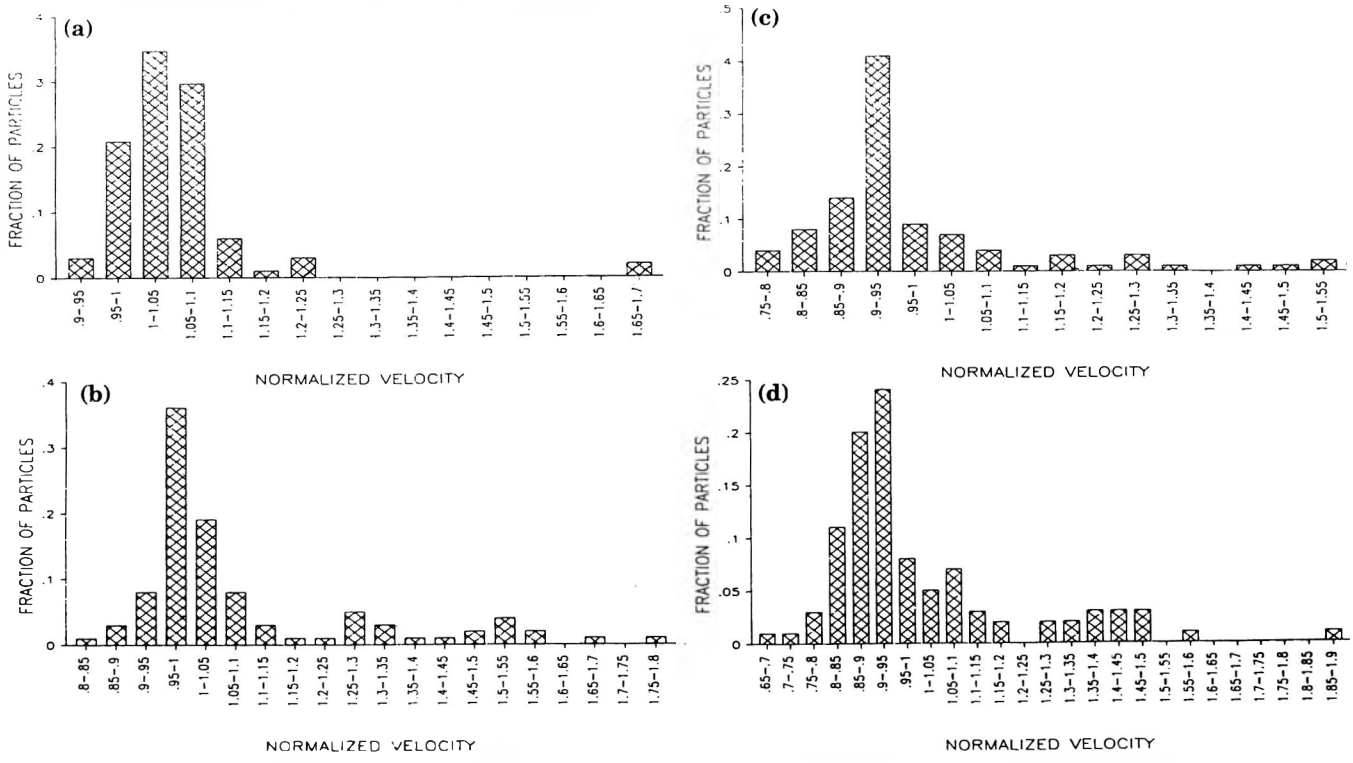

Fig. 5—Velocity distributions for 0.5% CMC carrier, 120 rpm pump speed and particle concentrations of (a) 0.2036%, (b) 0.4072%, (c) 0.6108%, and (d) 0.8145%.

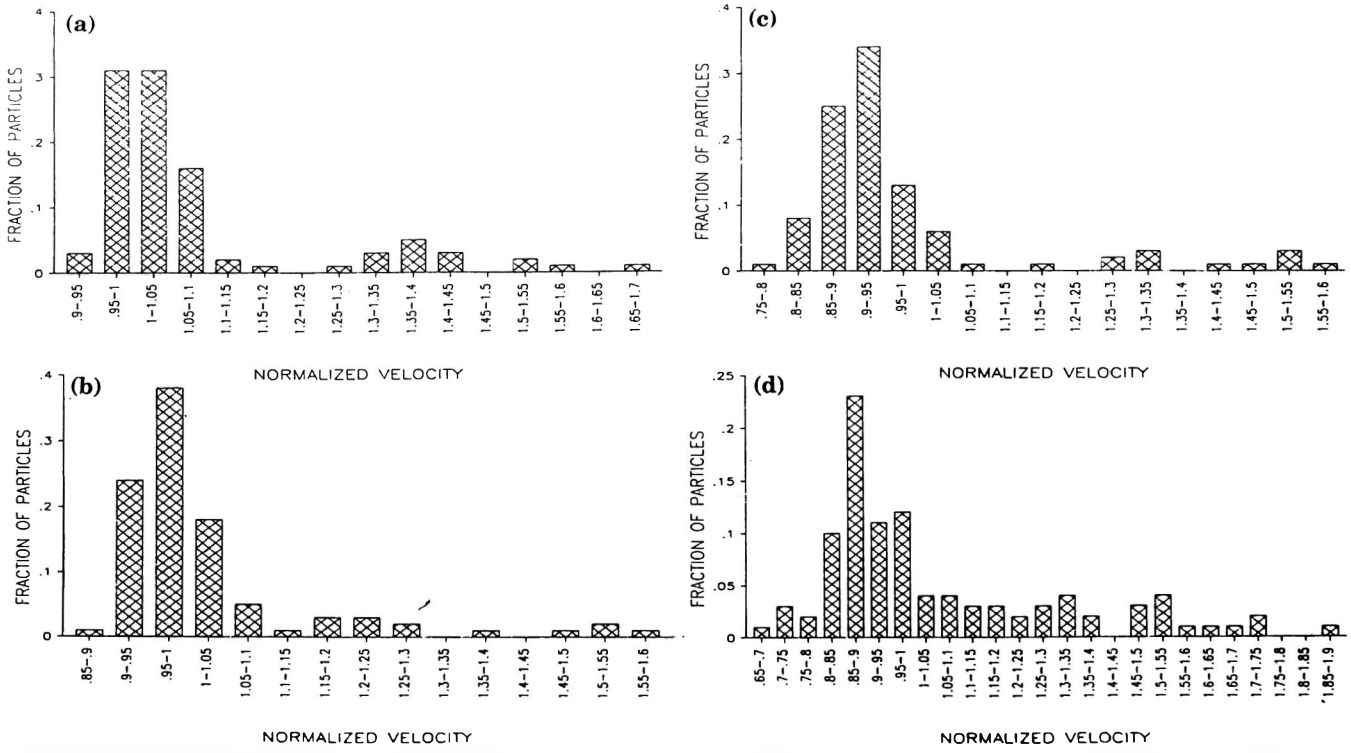

Fig. 6—Velocity distributions for 0.5% CMC carrier, 140 rpm pump speed and particle concentrations of (a) 0.2036%, (b) 0.4072%, (c) 0.6108%, and (d) 0.8145%.

significant outliers (fast particles) were observed though the majority were at relatively low values of V_n, indicating that overprocessing of a large proportion of particulates was likely to occur under such conditions. Fast-moving particles may have occurred because of density variations between individual particles.

At the highest viscosity (Fig. 7 to 9), values of V_n below 1 were rare. Under these conditions, although the majority of particles were only slightly off the bottom, they moved with relatively high V_n values. This may be due to the increasing steepness of the velocity gradient adjacent to the wall as the flow behavior index decreased. Particle concentration effects were also clearly definable as with 0.5% CMC solutions. At the higher particle concentrations, (both for

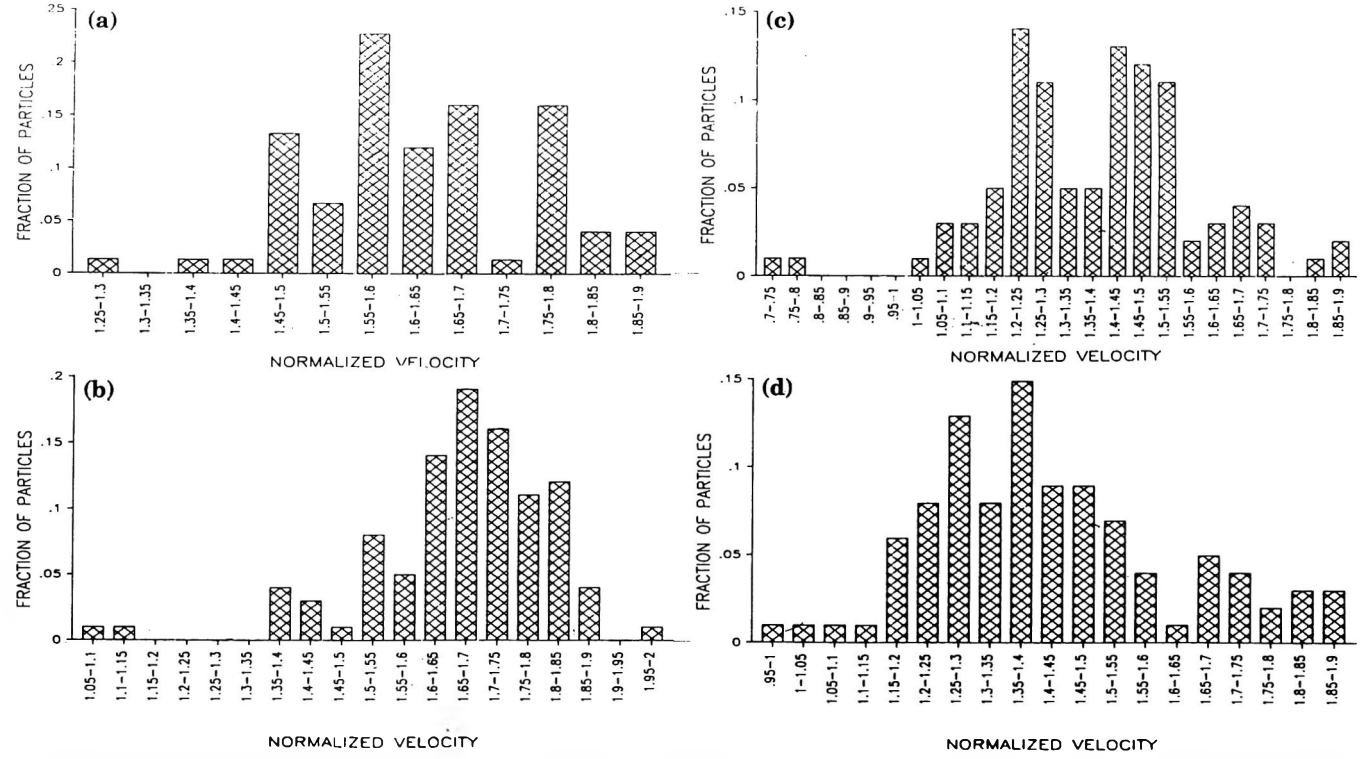

Fig. 7—Velocity distributions for 0.8% CMC carrier, 100 rpm pump speed and particle concentrations of (a) 0.2036%, (b) 0.4072%, (c) 0.6108%, and (d) 0.8145%.

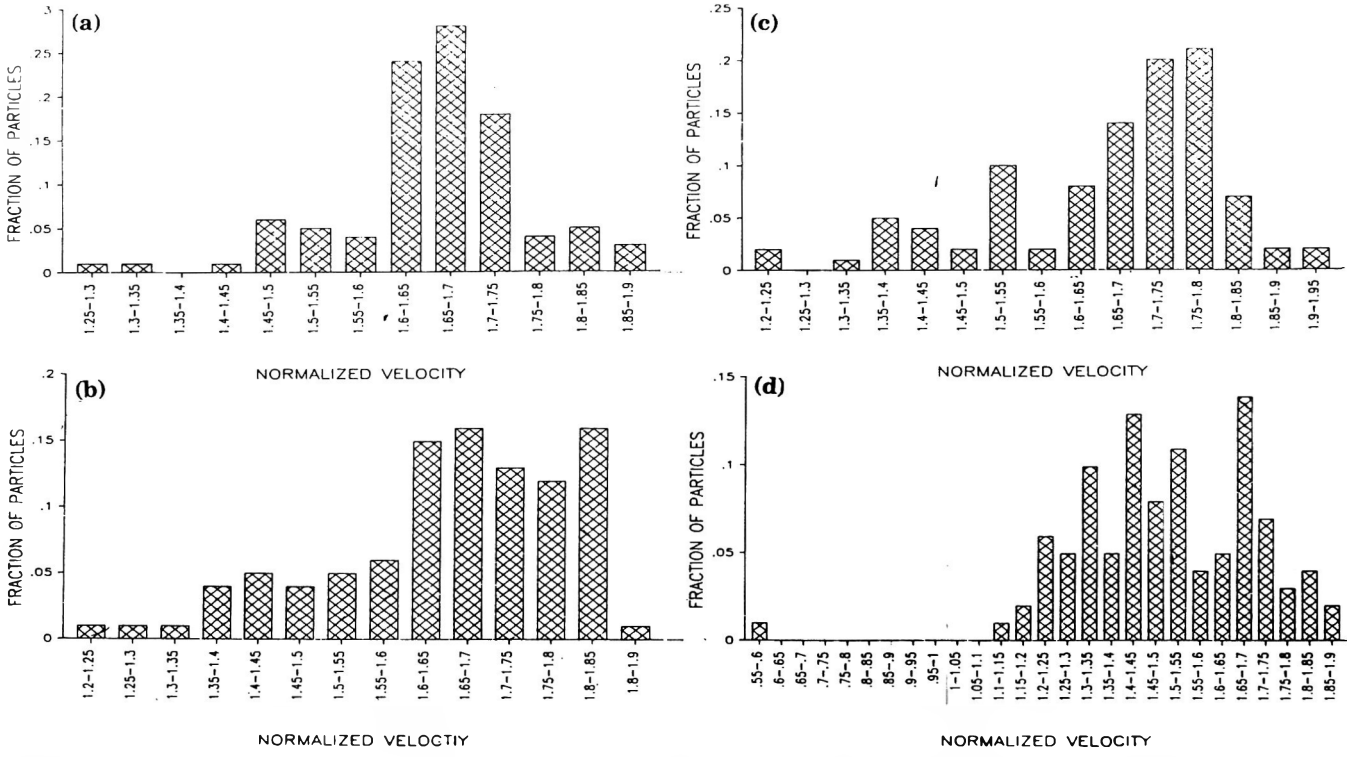

Fig. 8—Velocity distributions for 0.8% CMC carrier, 120 rpm pump speed and particle concentrations of (a) 0.2036%, (b) 0.4072% (c) 0.6108%, and (d) 0.8145%.

0.8% and 0.5% CMC solutions) particles were distributed throughout the tube cross section, although the concentration profile within the tube was not necessarily uniform (more particles at the bottom than the top). As expected, axially located particles moved fastest, and those adjacent to the walls (either bottom or top) moved slowest. Notably, at 0.8% CMC, high particle concentration, particle-particle hydrodynamic interactions tended to keep the majority of particles along a straight path.

Distribution models

The likelihood ratio test (Lawless, 1982) indicated that the distributions obtained would be best described by a log normal

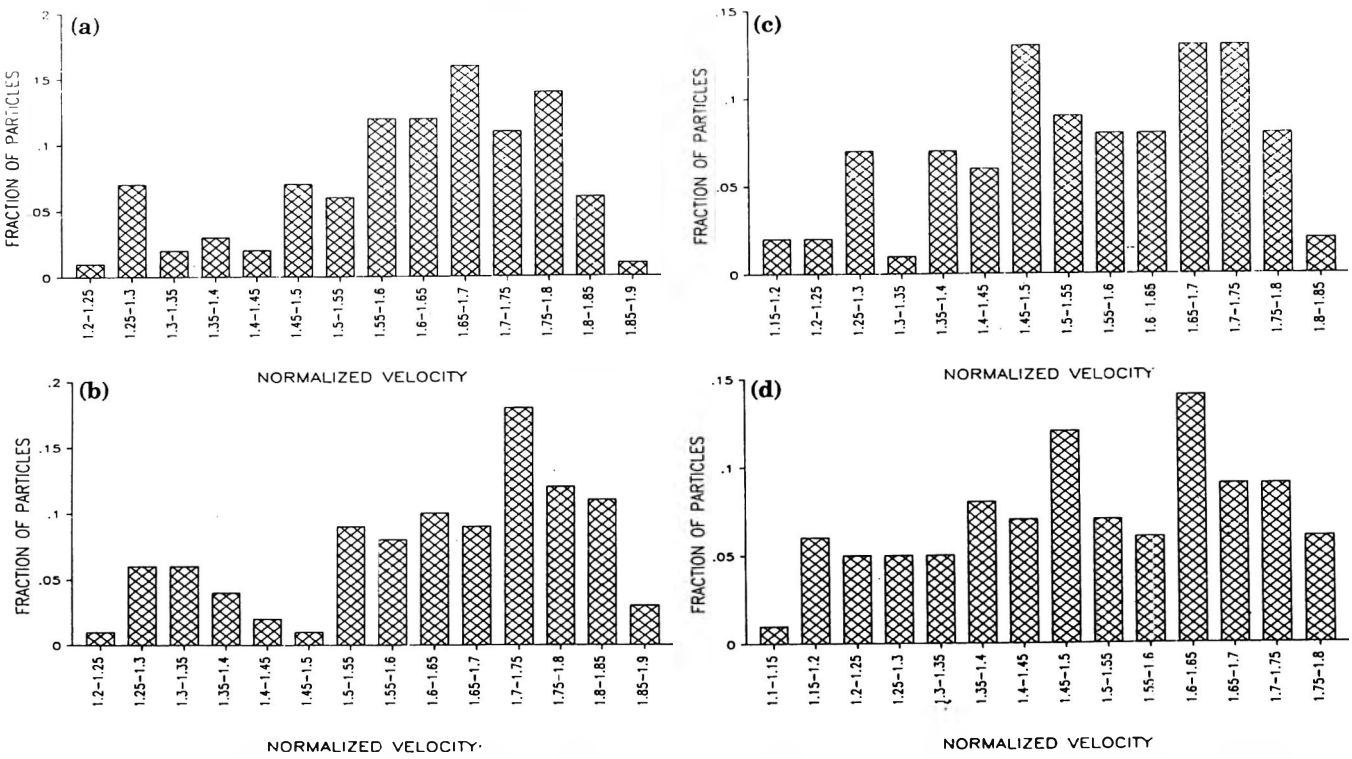

Fig. 9—Velocity distributions for 0.8% CMC carrier, 140 rpm pump speed and particle concentrations of (a) 0.2036%, (b) 0.4072%, (c) 0.6108%, and (d) 0.8145%.

Table 1—Means and standard deviations of the natural logarithms of normalized particle velocities (V_n) for 0.2% CMC carrier fluid

Particle conc (%)	Pump speed (rpm)	Statistical parameters of ln(V_n) Mean	Standard deviation
0.2036	100	−0.2738	0.1025
	120	0.0394	0.1453
	140	0.2089	0.1711
0.4072	100	−0.0897	0.2297
	120	0.0565	0.1611
	140	0.2458	0.1793
0.6108	100	0.1271	0.0991
	120	0.2046	0.1222
	140	0.1712	0.1571
0.8145	100	0.1440	0.1845
	120	0.1845	0.0965
	140	0.2922	0.1732

Table 2—Means and standard deviations of the natural logarithms of normalized particle velocities (V_n) for 0.5% CMC carrier fluid

Particle conc (%)	Pump speed (rpm)	Statistical parameters of ln(V_n) Mean	Standard deviation
0.2036	100	0.0425	0.0440
	120	0.0485	0.0853
	140	0.0723	0.1285
0.4072	100	0.0338	0.1171
	120	0.0670	0.1614
	140	0.0169	0.1168
0.6108	100	−0.0062	0.1644
	120	−0.0410	0.1406
	140	−0.4020	0.1496
0.8145	100	−0.0207	0.1930
	120	−0.0173	0.1841
	140	0.0225	0.2276

distribution. In this, the probability that a particle possesses a normalized velocity between x_1 and x_2 is given by the following probability density function (PDF):

$$P(x \leq x_2) - P(x \leq x_1) = F(x_2) - F(x_1) \qquad (1)$$

where F is a cumulative distribution function (CDF) given by:

$$F(x) = P(x \leq x) = P(z \leq [\ln x - \mu]/\sigma) \qquad (2)$$

where μ and σ are the mean and standard deviations, respectively, of the logarithms of the particle normalized velocities for a test run. z is the number of standard deviations from the mean at which the observation is located. The required probability can easily be evaluated using a standard normal distribution table with $\mu = 0$ and $\sigma^2 = 1$. Means and standard deviations of the logarithms of normalized velocities for each test run are summarized in Tables 1 through 3.

Comparisons were made between the actual and predicted values of the CDF of particle normalized velocities for each run. The actual values were derived from experimental data, while the predicted values were based on means and standard deviations of the logarithms of experimental data. Results indicated that the model fit the data well. The theoretical curves not only stayed within the 95% confidence limits of the experimental data, but they practically overlapped with these data at several zones and remained very close at most other zones.

Based on these studies and those of Dutta and Sastry (1989), it is feasible to consider development of complete models for velocity distributions based on the conditions of test. The means and standard deviations of the data can be characterized using the values of particle Froude number (Fr_p), nondimensional viscosity (μ_{ND}) and particle concentration (c) using the correlations of Dutta and Sastry (1990), and the distribution characteristics generated by the lognormal models of our current study. The principal limitations here are the conditions of study; the data being derived from experimental observation should not reasonably be extrapolated to conditions outside the test, particularly if particle size and shape change. Although modeling from first principles shows promise, quantitative as well as qualitative agreement will be required before they can become applicable. For this purpose, rigorous models of particle/fluid flow are necessary.

Table 3—Means and standard deviations of the natural logarithms of normalized particle velocities (V_n) for 0.8% CMC carrier fluid

Particle conc (%)	Pump speed (rpm)	Statistical parameters of ln (V_n) Mean	Standard deviation
0.2036	100	0.4840	0.0769
	120	0.5027	0.0624
	140	0.4737	0.1004
0.4072	100	0.5053	0.0952
	120	0.4995	0.0890
	140	0.4780	0.1116
0.6108	100	0.3125	0.1570
	120	0.5029	0.0947
	140	0.4377	0.1110
0.8145	100	0.3416	0.1339
	120	0.3945	0.1546
	140	0.3990	0.1214

Table 4—Values of normalized fastest-particle velocity and maximum theoretical normalized velocity (V_{nmax}) for each test condition

CMC conc (%)	V_{nmax}	Pump Speed (rpm)	Normalized fastest particle velocity Particle conc, c (%) 0.2036	0.4072	0.6108	0.8145
0.2	1.913	100	0.956	1.627	1.491	1.651
		120	1.670	1.570	1.634	1.767
		140	1.680	1.729	1.622	1.716
0.5	1.817	100	1.316	1.747	1.656	1.742
		120	1.691	1.763	1.531	1.853
		140	1.660	1.575	1.595	1.861
0.8	1.692	100	1.899	1.957	1.867	1.891
		120	1.870	1.890	1.921	1.850
		140	1.853	1.889	1.818	1.796

These observations and those we reported previously (Dutta and Sastry, 1990) suggest the most critical factor affecting particle velocity is viscosity of the carrier fluid. At low viscosities and flow rates, system design based on fastest particle velocity could result in overprocessing the majority of particles. At higher flow rates and viscosities, fewer particles would be overprocessed by this approach. The key restriction is that our studies have been performed (out of necessity) at low particle concentrations. The nature of particle-particle interactions changes greatly with particle concentration, as is well established from suspension rheology, with particle motion becoming increasingly restricted at higher concentrations, and playing a greater role in the behavior of the entire "fluid."

Fastest-particle velocities

Values of normalized fastest-particle velocities are summarized in Table 4, along with maximum theoretical normalized velocity (V_{nmax}) for the streamline at the tube axis. For each particular fluid, this is:

$$V_{nmax} = (3n + 1)/(n + 1) \quad (3)$$

where n is the flow behavior index for the fluid. Values for the present case were presented by Dutta and Sastry (1990).

In Table 4, normalized velocities for the 0.2% CMC solution were within the expected limiting value of V_{nmax}; however for 0.5% CMC, V_{nmax} was exceeded in two cases—for the highest particle concentration at the two highest flow rates. The principal explanation for such a trend appears to be channeling; i.e. a large number of slow-moving particles on the bottom of the tube would effectively cause a reduction of the effective cross-section through which flow occurs, thereby increasing the velocity of the remaining fluid and particles through the gap. Thus the fastest moving fluid particles could likely move faster than predicted by the flow behavior index, and carry particles at a higher velocity through the gaps. However, no attempts were made to determine maximum fluid velocity in these studies.

A more puzzling situation occurred for 0.8% CMC solutions, since the data in Table 4 indicated that V_{nmax} was exceeded in all cases. Inspection of Fig. 7 through 9 indicates that a significant fraction of particles exceeded V_{nmax}. Since the results were consistent regardless of concentration, channeling was not likely the sole explanation. One explanation might be that since particles were more uniformly distributed in the cross-section, they may have altered the effective rheological behavior of the composite "fluid" to an extent to which the classical power-law velocity profile no longer applied. If the particle interactions with fluid resulted in substantial changes in shear rate profile, the behavior of the fluid could conceivably be different from that expected from the power law assumption. Notably, none of the particle exceeded the Newtonian value of 2.0 for V_{nmax}. The possibility of error in measurement of mean fluid velocity was considered carefully, and dismissed because measurement errors and influence coefficients for measurement of the various parameters (mass, time and pipe inside diameter) were small. The possibility of variations in flow rate during the test was also ruled out due to the constancy of pump speeds measured by tachometry and the fact that pulsation or surge effects (easily observable in particle flow) were not observed visually during experiments or on videotape.

The above observations indicate that much remains to be learned about particle/fluid flow behavior that may have major influences on process design. Within the limits of experimental conditions, even for fluids exhibiting pseudoplastic behavior, a safety factor of 2.0 for the residence time may be necessary (the fastest particle observed in these studies had a V_n of 1.957), as required by the FDA. For most test conditions, the fastest particles moved at or close to the central axis of the tube, following relatively unobstructed paths. At much higher concentrations than those of this study, the likelihood of unobstructed passage for individual particles is highly unlikely, and true flow characteristics are not well understood. Additionally, the particles which are observed as fast-moving in one section may not necessarily be as fast in another. For these reasons, applications of these data should be limited to the range of experimental variables studied, particularly particle concentrations. Further, our present studies do not include effects of bends, where the profile may change depending on levels of experimental variables.

Particle-particle interactions and qualitative observations

An interesting observation was the "leapfrog" motion referred to earlier where a relatively fast-moving particle rolling along the bottom and approaching a slower-moving particle in front of it, would pass over and in front of the slower particle by what appeared to be a combination of wake, boundary layer and Magnus lift effects. Such effects have been described in saltation flows (Zandi, 1971). We noted that particles not only collided but interacted hydrodynamically by an attraction-repulsion mechanism that appeared to be caused by pressure changes in the interparticle gap. This observation was consistent with those of Davis et al. (1986), who have shown that a significant pressure pulse exists between particles that approach one another within a liquid. They showed deformation of particles began due to the pressure pulse before collision occurs. At the lowest concentrations, particles interacted hydrodynamically in a manner described by Segré (1965), by forming "necklaces" or strings of particles which changed relative position only slightly in a slow oscillatory fashion. Sometimes two or three particles (four to five at higher concentrations) would form clusters, which could be broken up on collision with a fast-moving particle or by "leapfrog" action.

Some particles changed velocity during passage through the tube, depending on radial position and interaction with other

particles. In some cases, acceleration effects were highly significant, as particles leaped over others, and decelerations occurred due to settling and collisions with slow-moving clusters. Results presented here reflect the velocity of particles over a relatively greater length. The action over an entire length of holding tubes with multiple bends merits further investigation.

CONCLUSIONS

WITHIN CONDITIONS of the test, velocity distributions of particle suspensions in holding tube flow were represented by log normal distributions. Viscosity was the strongest factor affecting the distribution of velocities. At low viscosities and flow rates, where the majority of particles lag the fluid, a fastest-particle process design strategy would likely overprocess the majority of particles. At low-viscosity conditions, the fastest-particle velocities were found to be within the centerline velocity of a pseudoplastic fluid; however, at higher viscosities, this velocity was exceeded. This occurred occasionally at an intermediate viscosity and consistently at the highest viscosity. No particles were observed to move faster than twice the mean fluid velocity.

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RADIOLYTIC DEGRADATION OF SORBIC ACID. . .From page 1702

of oil, pH of the system and nature of additives. While vegetable oils had protective action both in chapaties and in isolated systems, sugars had catalytic action in isolated systems. In chapaties neither salt nor sugars significantly influenced rate of degradation. SA was more susceptible to radiolytic degradation at lower pH.

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A Method to Determine Initial Freezing Point of Foods

JORGE SUCCAR and KAN-ICHI HAYAKAWA

ABSTRACT

An accurate initial freezing point T_{sh} is required for reliable analysis of a food freezing or thawing process. Two previous formulae for accurately estimating enthalpies, one for temperatures above T_{sh} and another below T_{sh}, were used for this development. The estimation of T_{sh} was based on the mathematical formulation of continuity in food enthalpies estimated by both formulae at T_{sh}. The method was validated by estimating T_{sh} values of aqueous solutions of sucrose and NaCl at different solutions. There was fair agreement in the T_{sh} values of fresh fruit and vegetables, lean beef meat and lean fish meat estimated by the proposed and a previous method. One potential advantage of our new method was the direct estimation of T_{sh} through analysis of food enthalpies collected at different temperatures.

INTRODUCTION

WATER in food contains solutes. The food freezing proceeds in a temperature-range, starts to freeze at a specific temperature and is completed at a lower temperature. Several researchers (Hayakawa and Bakal, 1973; Hayakawa et al., 1983; Heldman and Singh, 1981; Hsieh et al., 1977; Nonino and Hayakawa, 1986; Succar and Hayakawa, 1984) found the initial freezing point of food, T_{sh}, influenced greatly the freezing or thawing times of foods. T_{sh}, therefore, should be detemined accurately for reliable analysis of food freezing or thawing.

Heldman (1974) developed semitheoretical formulae to estimate T_{sh} of food through thermodynamic analysis. For that analysis, the liquid was assumed to be a binary solution to which Clausius-Clapeyron equation was applied. The approach was further refined by Chen (1985), who obtained the following equation for estimating T_{sh}:

$$H_{sh} = (T_{sh} - T_r)\{0.37 + 0.3\, x_{so} + 7.45836 \cdot 10^4\, R/(M_s\, T_{sh}\, T_r)\} \quad (1)$$

An effective molecular weight, M_s, in the above equation is dependent on food and its moisture content. The M_s values of selected foods were estimated by:

$$M_s = b/(1 + a\, X_{so}) \quad (2)$$

Many foods are in a liquid form. Chen (1986) and Chen and Nagy (1987), obtained formulae applicable to ideal and nonideal solutions through a similar analysis. In addition, they obtained cubic regression formulae to estimate T_{sh} by curve-fitting published T_{sh} data of different aqueous binary solutions. These regression formulae estimate T_{sh} very accurately. They, may be used to examine reliabilities of predictive methods applicable to binary solutions. Chen (1988) also published formulae applicable to three different orange juices and analyzed the T_{sh} values of glycerol, aqueous solutions (Chen, 1987).

Chang and Tao (1981) analyzed statistically published T_{sh} data as a part of their study on food enthalpy and obtained the following regression equations.

For meat:

$$T_{sh} = -1.92 + 1.47\, X_{wo} \quad (3)$$

For fruits and vegetables:

$$T_{sh} = 14.46 - 49.19\, X_{wo} + 37.07\, X_{wo}^2 \quad (4)$$

For juice:

$$T_{sh} = -152.63 + 327.35\, x_{wo} - 176.49\, X_{wo}^2 \quad (5)$$

One approach for estimating T_{sh} is through the mathematical analysis of enthalpy data. Jason and Lang (1955) were the first researchers to develop such a method. They differentiated numerically a curve obtained by plotting enthalpies against temperature for determining the apparent specific heat of fish muscle. They estimated T_{sh} by locating the temperature of the maximum derivative. They assumed continuity in the derivative at temperatures around T_{sh} for their estimation although this was not a valid assumption. At temperature below T_{sh}, a functional relationship between temperature and enthalpy is governed by the latent heat of phase change and by the sensible heat of ice, unfrozen solution and solids in food. On the other hand, at temperatures above T_{sh}, the relationship is influenced by the sensible heat of unfrozen solution and solids only. There is, therefore, a discontinuity in the derivative at T_{sh}. Because of the assumed continuity in the derivative, they obtained unrealistically large, specific heats at temperatures above T_{sh}. They stated that "high apparent specific heat in the region which extends for several degrees (°C) above the freezing points remains unexplained".

A reliable mehtod for directly estimating T_{sh} using food enthalpy data would be useful to researchers since enthalpy data may be determined easily and accurately. Our objective was to develop such a method.

MATHEMATICAL DEVELOPMENT

EMPIRICAL FORMULAE were developed previously for accurately estimating food enthalpies as a function of temperature (Succar and Hayakawa, 1983). These formulae are based on the modification of thermodynamically derived theoretical formulae (Schwartzberbg, 1976). They were used to estimate T_{sh}.

Equation 6 estimates food enthalpy when $T > T_{sh}$.

$$H = C_l\, T + H_o \text{ for } T \geq T_{sh} \quad (6)$$

In the above equation, C_l is the specific heat of the unfrozen food, T is food temperature expressed in °C, and H_o is the value of H at 0°C. When $T \leq T_{sh}$, Eq. (7) estimates accurately H as a function of T.

$$H = c_e\,(T - T_r) + \frac{D}{n-1}\left[\frac{1}{(T_{sw} - T)^{n-1}} - \frac{1}{(T_{sw} - T_r)^{n-1}}\right] + H_c \text{ for } T \leq T_{sh} \quad (7)$$

In Eq. (7), T_r is a reference temperature for enthalpy determination, and c_e, D, n and H_c are empirical constants which may be determined by a nonlinear parameter estimation technique using enthalpy data (Metzler et al., 1976). The symbol T_{sw} represents the freezing point of pure water (0°C).

Note that the first derivatives estimated by Eq. (6) and (7) have different values at T_{sh}. However, Eq. (6) has a well-defined first derivative for any temperature and eq. 7 for any temperature below T_{sw} (0°C).

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As previously indicated, dH/dT is discontinuous at T_{sh}. However, the values of enthalpies estimated by Eq. (6) and (7) should be identical at T_{sh}, Eq. (8).

$$c_l T_{sh} + H_o = c_e(T_{sh} - T_r) + \frac{D}{n-1}\left[\frac{1}{(T_{sw} - T_{sh})^{n-1}} - \frac{1}{(T_{sw} - T_r)^{n-1}}\right] + H_c \quad (8)$$

Through rearrangement of Eq. (8) and by replacing T_{sw} = O°C, the following equation was obtained:

$$T_{sh} - \frac{|T_{sh}|^{(1-n)}}{Z_1} - Z_2 = 0 \quad (9)$$

where:

$$Z_1 = (n - 1)(c_l - c_e)/D \quad (10)$$

$$Z_2 = \frac{1}{(c_e - c_l)}\left[c_e T_r + \frac{D}{(n-1)|T_r|^{n-1}} - H_c + H_o\right] \quad (11)$$

The value of T_{sh} in °C may be estimated by finding the largest negative root (the smallest absolute value) of Eq.(7). Any reliable numerical method may be used to solve Eq. (9) since it is nonlinear. Newton-Raphson's method (Hildebrand, 1956) was used for our work. With this method, an approximate T_{sh}, $T_{sh,k}$, is refined by:

$$T_{sh,k+1} = T_{sh,k} - f(T_{sh,k})/f'(T_{sh,k}) \quad (12)$$

In Eq. (12), $f(T_{sh,k})$ represents the left side of Eq. (9). Its first derivative, $f'(T_{sh,k})$, is:

$$f'(T_{sh,k}) = 1 + (1 - n)/(Z_1|T_{sh,k}|^n) \quad (13)$$

Substituting Eq. (9) and (13) into Eq. (12), we obtained:

$$T_{sh,k+1} = T_{sh,k} - (T_{sh,k} - |T_{sh,k}|^{1-n}/Z_1 - Z_2)/\{1 + (1 - n)/(Z_1|T_{sh,k}|^n)\} \quad (14)$$

The iterative refinement of T_{sh} is continued intil a difference in successfully estimated T_{sh} becomes less than a specified value, ϵ (e.g. 0.005C°).

$$|T_{sh,k+1} - T_{sh,k}| < \epsilon$$

We need to assume the first approximate T_{sh} value, T_{sh1}, since Eq. (13) is not self-starting. This value should be any temperature below 0°C since Eq. (7) satisfies all requirements for use of Newton-Raphson method at this temperature according to our numerical analysis of available enthalpy curves. For most cases, −1.0°C is suitable as T_{sh1}.

MATERIALS & METHODS

Accurate data are available on the T_{sh} of sucrose and sodium chloride aqueous solutions (Weast, 1985). Values of T_{sh} for these solutions, were estimated by the proposed method for validation. Enthalpy values (cal/g of solution) of sucrose solutions with moisture ranging from 0.65 (g water/g solution) to 0.90 were collected from a Mollier chart prepared for a T_r value of −40°C (Burke, 1954).

For each moisture level, over 15 enthalpies were obtained in a nonfreezing temperature range and a similar number of enthalpies in a freezing temperature range (i.e. over 30 enthalpies/moisture level. Less enthalpy data would be sufficient to accurately estimate empirical parameters). These enthalpies were obtained through the cubic spline interpolation (Ahlberg et al., 1967) of enthalpies on isothermal lines in the chart. The values of C_l and H_o in Eq. (6) were obtained from enthalpies at nonfreezing temperatures through a linear regression analysis. The values of parameters included in Eq. (7) (C_e, D, n and H_c) were determined from remaining enthalpies through the nonlinear parameter estimation technique. The estimated parameters were used to determine T_{sh} values. The T_{sh} of sodium chloride solutions of five different concentrations were estimated also through similar calculations by using a published Millier chart (Benzler, 1955).

Riedel (1950) obtained Mollier charts for fruits and vegetables, lean beef meat (Riedel, 1957a), egg white and egg yolk (Riedel, 1957b) and lean fish meat (Riedel, 1956). The T_{sh} values of these foods were estimated using the proposed method and formulae developed by Chen (1985), Eq. (1), and by Chang and Tao (1981), eqs. 4 and 5. These values were obtained also directly from the charts for comparison.

To apply Eq. (1) enthalpies at T_{sh} (H_{sh}) were obtained directly from the above mentioned Mollier charts since they were required. The equation was transformed to a quadratic equation with respect to T_{sh}. The transformed equation was solved analytically to obtain T_{sh}. One proper quadratic root (the larger root) was chosen from two roots. Equation (1) was not used to estimate T_{sh} values of egg yolk and egg white since their effective molecular weights were not available. No regression formulae were provided for egg yolk and white by Chang and Tao (1981). Note also that both Eq. (4) and (5) were used to estimate the T_{sh} of fruits and vegetables because no limitation in applicable X_{xo} range was reported.

RESULTS & DISCUSSION

TABLE 1 shows the T_{sh} values of sucrose and sodium chloride aqueous solutions, determined by the proposed method, and the formulae were obtained by Chen and Nagy (1987) directly from the published Mollier charts (Benzler, 1955; Burke, 1954). Among the tabulated, those estimated by the Chen and Nagy's (1987) formulae are considered most accurate since they obtained thorough curve-fitting of published T_{sh} data. According to the authors, the errors are less than 0.7% for T_{sh} of sucrose solutions and less than 0.2% for T_{sh} of salt solutions. There was close agreement in T_{sh} values by all three methods.

Table 2 shows the T_{sh} values of five different foods estimated using formulae derived by Chang and Tao (1981), and by Chen (1985), using the proposed method, and directly from the published Mollier charts (Riedel, 1950, 1956, 1957a, 1957b). The following observations were made from Table 2. With fresh fruit and vegetables, T_{sh} values estimated by Chen's formula and proposed methods were closer to the Mollier chart values as compared to those estimated by the Chang and Tao's formula. On the other hand, the Chang and Tao's method estimated the T_{sh} values of lean beef meat slightly nearer chart values than those estimated by the other two methods. The T_{sh} values estimated by these two methods were reasonably close to each other. Note the chart value at moisture 0.70g water/g food was identical to the chart value at 0.63g water/g food. The identical T_{sh} at the two different moisture contents is unreasonable. There could be errors, in the chart values of lean beef meat. With lean fish meat, there were similar differences between estimated T_{sh} values and chart values for all three methods although those estimated by Chen's formula and our proposed method were reasonably close. With egg yolk and egg white, T_{sh} values obtained from the charts and estimated by the proposed method were in fair agreement.

One advantage of our proposed method, compared to the

Table 1—Validation of proposed method

Aqueous solution	Moisture content (g/g)	T_{sh}(°C) Determined by following methods		
		Chen[a]	Proposed	Mollier Charts[b]
Sucrose	0.90	−0.63	−0.60	−0.6
	0.85	−1.00	−1.02	−1.0
	0.80	−1.45	−1.50	−1.5
	0.75	−1.99	−2.02	−2.0
	0.70	−2.66	−2.67	−2.7
	0.65	−3.48	−3.50	−3.5
NaCl	0.98	−1.19	−1.22	−1.2
	0.96	−2.42	−2.52	−2.4
	0.94	−3.70	−3.71	−3.8
	0.90	−6.55	−6.67	−6.6
	0.86	−9.94	−9.93	−10.0

[a] These were obtained using the following formulae obtained by Chen and Nagy (1987).
$T_{sh} = -6.001 X_{so} + 0.419 X^2_{so} - 33.292 X^3_{so}$ for sucrose solutions.
$T_{sh} = -59.278 X_{so} - 7.332 X^2_{so} - 544.427 X^3_{so}$ for NaCl solutions.
[b] Benzler (1955) and Burke (1954).

Table 2—Comparison of T_{sa} values estimated by different methods

Food	Moisture Content (g/g)	T_{sh} (°C) estimated by the following methods: Charts[a]	Chang & Tao (1981)	Chen[e] (1985)	Proposed
Fruit & vegetable (juice)	0.96	−0.4	1.50[b](−0.93)[c]	−0.40	−0.39
	0.87	−1.4	−0.18 (−1.32)	−1.41	−1.38
	0.75	−3.3	−1.48 (−6.29)	−3.28	−3.19
	0.61	−7.0	−1.65 (−18.52)	−6.86	−6.98
Lean beef meat	0.74	−0.6	−0.73[d]	−1.00	−0.99
	0.70	−1.0	−0.79[d]	−1.20	−1.01
	0.63	−1.0	−0.89	−1.63	−1.76
Lean fish meat	0.82	−0.1	−0.61	−0.87	−0.80
	0.75	−1.0	−0.72	−1.29	−1.00
	0.66	−1.3	−0.85	−1.96	−1.95
Egg yolk	0.50	−0.01			−0.12
	0.40	−0.3			−0.41
	0.30	−1.1			−1.03
	0.20	−2.3			−2.65
Egg white	0.90	−0.01			−0.01
	0.80	−0.05			−0.16
	0.70	−0.6			−1.00
	0.60	−1.0			−1.48
	0.50	−2.8			−3.14
	0.40	−5.0			−5.28

[a] Mollier charts (Riedel, 1950, 1956, 1957a, 1957b).
[b] Estimated by Eq. (4) for fruits and vegetables.
[c] Estimated by Eq. (5) for juice (values enclosed with parentheses).
[d] Estimated by Eq. (3).
[e] Estimated by Eq. (1).

published procedure, was that T_{sh} might be estimated directly through analysis of experimental enthalpy data. The method is likely applicable to any food on which accurate enthalpy data are available.

NOMENCLATURE

- a,b — Empirical constants for estimating effective molecular weight. a = −1 for lean beef and cod muscle and a = 0.25 for orange and apple juices. The b values of lean beef, cod muscle and juices are 535.4, 404.9, and 200, respectively according to Chen (1985). (−), (g/g mol).
- c_e, c_l — Empirical constants in Eq. (7) and (6), respectively, (cal/g).
- D — Empirical constant in Eq. (7) (cal C^{on-1}/g).
- H — Enthalpy of food (cal/g).
- H_c, H_o — Empirical constants in Eq. (6) and (7), respectively (cal/g).
- M_s — Effective molecular weight of unfrozen solution (g/g mole).
- n — Empirical constant in Eq. (7) (−).
- R — Universal gas constant [cal/(mole °K)]
- T_r — Reference temperature used to determine food enthalpy.
- T_{sh} — Initial freezing point of food (°C).
- T_{sw} — Freezing point of pure water (°C).
- X_{so} — Initial weight fraction of solute (−).
- X_{wo} — Initial weight fraction of water (−).
- Z_1, Z_2 — Expressions defined by Eq. (10) and (11), respectively.
- ϵ — Converging criteria.

Subscript

- k — k-th iterative estimation of T_{sh}.
- k+1 — (k+1) st iterative estimation of T_{sh}

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Mutagenicity and Identification of the Reaction Products of Aqueous Chlorine or Chlorine Dioxide with L-Tryptophan

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ABSTRACT

Nonvolatile products generated from reactions of graded molar ratios of aqueous chlorine or chlorine dioxide with L-tryptophan (1:1, 3:1 and 7:1) were shown to be direct-acting mutagens to *Salmonella typhimurium* TA100 and TA98. Increasing the ratio of disinfectant relative to amino acid led to increased mutagenic activity, with mutagenicity highest at the 7:1 molar ratio. Several fluorescent bands obtained after thin layer chromatographic fractionation of the reaction mixtures were shown to be more mutagenic than the reaction mixtures. GC/MS analysis of the compounds in a highly mutagenic fraction of the aqueous chlorine reaction products identified 1,1,3-trichloropropanone, 1,1,3,3-tetrachloropropanone and dichloroquinoline.

INTRODUCTION

CHLORINE is used worldwide as a disinfectant for control of microbes in water and for cleaning and sterilizing food and food processing equipment. The use of chlorine for potable water treatment has improved the quality of life and prevented transmission and spread of waterborne diseases. However, safety concerns were raised when chlorine was reported to react with organic matter in water to produce chlorinated (e.g. trihalomethanes, THM) and/or oxidized compounds, some of which are carcinogenic and/or mutagenic (Bellar et al., 1974; Rook, 1974). These concerns were exacerbated when chloroform, the major THM, was found carcinogenic in test animals (NCI, 1976). Fulvic and humic substances in water as well as amino acids, peptides and proteins have been implicated and confirmed as precursors of chlorination reaction products in drinking water (Bierber and Trehy, 1983; Christman et al., 1980; Oliver, 1983; Trehy et al., 1986).

Mutagen formation has been reported from extracts of chlorinated drinking water (Loper, 1980), fulvic and humic acids (Bull and Robinson, 1985; Meier et al., 1983; Nazar and Rapson, 1982) and amino acids (Horth et al., 1987; Rapson et al., 1985; Sussmuth, 1982). Under water treatment conditions chlorine reacts with a vast array of compounds with different physical and chemical properties. This makes difficult the isolation, separation and identification of reaction products.

Unlike aqueous chlorine, use of chlorine dioxide in water treatment has been reported to form little or no THM (Symons et al., 1981). Chlorine dioxide is widely used in Europe for water treatment and is being considered as an alternative to chlorine for drinking water treatment in the United States (USEPA, 1983). Even though chlorine dioxide does not produce THM in drinking water, we were concerned about the possible health hazards associated with its endproducts, chlorate and chlorite (Abdel-Rahman et al., 1984; Condie, 1986). Mutagenic activity has been detected in concentrates from chlorine dioxide-treated drinking water (de Greef et al., 1980; Kool et al., 1985).

Because organic mutagens may be produced in very minute quantities (μg/L levels) in drinking water, concentration of drinking water samples is necessary to obtain adequate quantities for mutagenicity studies. Several methods including reverse osmosis, liquid-liquid extraction, resin or activated carbon adsorption and freeze drying have been used for concentration. In our study liquid-liquid extraction (LLE) and Amberlite XAD resin adsorption methods were compared for effectiveness in concentrating reaction products in model reaction mixtures of aqueous chlorine or chlorine dioxide with tryptophan.

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MATERIALS & METHODS

Chemicals

L-Tryptophan was purchased from Sigma Chemical Company (St. Louis, MO). Radiolabelled L-tryptophan (side chain-3^{14}C, specific activity 53.76 mCi/mmole) was obtained from New England Nuclear, Inc. (Boston, MA). Amberlite XAD-2 and XAD-8 resins were purchased from Rohm and Haas (Philadelphia, PA). The resins were washed sequentially by continuous Soxhlet extraction with acetone and methanol, and stored in methanol. Prior to use, the resins were batch rinsed with distilled water and slurry-packed in a glass column. Silica gel G plates and all organic solvents were obtained from Fisher Scientific (Fair Lawn, NJ).

Preparation of aqueous chlorine and chlorine dioxide

Chlorine demand-free water was prepared by passing distilled water through a column containing Porapak Q (Waters Associates, Milford, MA). This water was used for preparation of all reagents and substrates. Aqueous chlorine was prepared by dropwise addition of 20 mL of 3M HCl to 6g of potassium permanganate (Fisher Scientific) in a closed system (Ghanbari et al., 1983). The chlorine gas generated was trapped in ice-cold chlorine demand-free water.

Chlorine dioxide was prepared by slow addition of 10 mL of a 2N H_2SO_4 solution to 6g of sodium chlorite (Eastman Kodak Co., Rochester, NY) in 6 mL water in a closed system (APHA, 1985). The chlorine dioxide gas generated was passed through a sodium chlorite column to remove traces of contaminants, (such as chlorine), and trapped in ice-cold water. The available chlorine and chlorine dioxide concentrations were checked by the iodometric titration method (APHA, 1985). Stock chlorine and chlorine dioxide solutions were diluted when necessary and used immediatley after preparation. All reagents were stored in the dark.

Reaction of aqueous chlorine or chlorine dioxide with tryptophan

Three molar ratios of aqueous chlorine or chlorine dioxide (1, 3 and 7) were selected to react with tryptophan in 0.1M sodium phosphate buffer (pH 7.0). For the 1:1 and 3:1 reactions, 14 mM tryptophan solutions (2.86g tryptophan/L) were reacted with 14 and 42 mM of each disinfectant, while the 7:1 reactions were carried out using 10 mM tryptophan solution (2.04g tryptophan/L) and 70 mM chlorinating agents. The reactions were conducted using equal volumes of reagent and substrate, therefore final concentrations were halved. Reactions were carried out by continuous mixing in covered glass jars at ambient temperature for 24 hr.

Blanks containing only tryptophan, aqueous chlorine or chlorine dioxide were also prepared the same way. After the reaction, any available chlorine or chlorine dioxide remaining was quenched by addition of small quantities of sodium sulfite. Samples were then filtered using a 0.45 μm membrane filter (Gelman Sciences, Inc., Ann Arbor, MI) to remove any precipitate. After the samples were split into two portions, the pH of one-half was adjusted to 2.5 by adding 85% phosphoric acid for Amberlite XAD resin adsorption while the other half was maintained at the original pH (7.0) for liquid-liquid extraction.

Liquid-liquid extraction (LLE)

The reaction mixtures containing different molar ratios of disinfectant and amino acid were extracted vigorously with ethyl ether, (previously shown to efficiently extract the reaction products (Trehy et al., 1986)), by mixing on a stirrer with magnetic bar about 24 hr. The phases were separated by separatory funnel and the aqueous phase discarded. The ethyl ether was removed by rotary evaporation *in vacuo* to leave a small residue quantitatively transferred into preweighed teflon-capped vials and dried under a gentle stream of nitrogen. The dried extracts were weighed, dissolved in and diluted with spectrometric-grade dimethyl sulfoxide (DMSO, Schwarz/Mann Biotech, Cleveland, OH) and used for mutagenicity evaluation.

Amberlite XAD resin adsorption

Amberlite XAD-2 resin was placed in a glass Column with an equal amount of Amberlite XAD-8 in a layer on top of it (column: 52 × 1.9 cm i.d.; height 30 cm). The acidified reaction mixture (pH 2.5) and all control solutions were passed twice through the resin column at about 40 mL/min. After most of the mixture had passed through and the liquid level was at the top of the resins, acetone was added and allowed to flow freely until it reached the bottom of the column. Flow was stopped and the acetone allowed to stand with the resins 10-15 min. A total of 300 mL acetone was passed through to elute the column, which was further eluted with 300 mL methanol.

The acetone and methanol eluates were concentrated separately by rotary evaporation *in vacuo* and the dried samples were weighed, and dissolved in DMSO. With acetone eluates, there was usually residual water. These samples were diluted in chlorine demand-free water (20 mL final volume) and filtered aseptically. The acetone and methanol eluates were separately assayed for mutagenicity.

Product distribution in the Amberlite XAD eluates

To the 10 mM solution of tryptophan in 500 mL sodium phosphate buffer (pH 7.0) was added [^{14}C]tryptophan (0.1 mCi) in 0.1 mL of the buffer. The radioactivity was determined by counting aliquots (0.1 mL) in 15 mL of Scintiverse II solution (Fisher Scientific) using a Beckman Scintillation spectrometer (Model LS 2800, Beckman Instruments, Inc., Fullerton, CA). An equal volume of 10 or 70 mM solution of aqueous chlorine or chlorine dioxide was added and reacted in the dark for 24 h at ambient temperature. The solution was filtered to remove precipitate and radioactivity of the filtrate was measured. Following acidification to pH 2.5, the aqueous mixture was concentrated by Amberlite XAD-2/8 adsorption as described.

The adsorbed compounds were successively eluted with acetone and methanol (300 mL each). The eluates were concentrated by rotary evaporation to known volume and the radioactivity measured. Aliquots of the radioactive acetone eluate were spotted on Analtech silica gel G plates (Fisher Scientific) previously heated 1 hr at 150°C, and developed 1 hr in the dark in a freshly prepared solvent system comprising hexane:ehtyl ether:acetic acid (50:50:2, v/v) for the aqueous chlorine products and hexane:ethyl ether:methanol:acetic acid (63:30:7:2, v/v) for chlorine dioxide reaction products. Several fluorescent spots separated; at each spot the silica gel was scraped and the radioactivity determined.

Eractionation of the reaction products

The acetone eluates of Amberlite XAD-2/8 concentrates from the 7:1 aqueous chlorine or chlorine dioxide:tryptophan reactions were subjected to TLC. Under long-wavelength UV light, several fluorescent bands were observed. The fluorescent bands were scraped off, extracted with acetone and filtered through Whatman No. 4 filter paper. The filtrate was concentrated by rotary evaporation *in vacuo*, weighed and dissolved in DMSO for mutagenicity evaluation.

Ames Salmonella/mammalian microsome assay

The mutagenicities of all extracts and TLC fractions were determined using the Ames plate incorporation assay in *Salmonella typhimurium* strains TA98 and TA100 (Maron and Ames, 1983). The assays were performed with and without the liver S9 mix prepared from rats pretreated with Aroclor 1254. The assay was conducted by adding 0.1 mL of overnight tester cultures with the test chemical and 0.5 mL of S9 mix or phosphate buffer into 2.5 mL of top agar containing small quantities of histidine and biotin. Concurrent positive and negative controls were included in all assays; 2-aminofluorene was used for both bacterial strains in the presence of the S9 mix. Methyl methanesulfonate was used for strain TA 100 and 2-nitrofluorene for TA98 when the S9 mix was not included. The sample was vortex mixed, poured onto minimal media agar plates and incubated at 37°C for 48 hr before counting colonies. For statistical analysis, the mutagenicity ratio, i.e., the number of revertants from test plates (spontaneous + induced) divided by controls (spontaneous) was used. Samples with a mutagenicity ratio of 2 or more were considered positive (Ames et al., 1975).

Gas chromatograph/Mass spectrometer/Data system

A Finnigan Instruments (San Jose, CA) model 4500 quadrupole mass spectrometer/gas chromatograph/data system (Incos Rev. 5.4) was used to identify some mutagens in the concentrates. A DB-5 capillary column (30 m × 0.32 mm i.d.; J. and W. Scientific, Rancho Cordova, CA) was programmed linearly from 50 to 300°C at 12°C/min with helium carrier gas at 25 mL/min. Chromatograms were run in both positive chemical ionization and electron impact (70 eV) modes.

RESULTS & DISCUSSION

A DARK-COLORED MIXTURE and precipitate were produced upon reaction of both aqueous chlorine and chlorine dioxide with tryptophan at all three ratios. The intensity of the color was greater for lower than for the highest ratio samples. As previously reported, the precipitate found to dissolve in DMSO (Kirk and Mitchell, 1980; Sen et al., 1989; Tan et al., 1987) was not mutagenic to either tester strain of *Salmonella* (data not shown).

Liquid-liquid extraction

The aqueous chlorine-tryptophan reaction mixtures after LLE with ethyl ether were mutagenic to strain TA98 in absence of S9 mix at the 1:1 and 7:1 ratios but not at the 3:1 ratio (Table 1). The presence of weakly mutagenic reaction products in the 3:1 sample may have contributed to lack of mutagenic activity of that sample. In strain TA100, mutagenic activity was only in the 7:1 ratio (Table 1). The 3:1 and 7:1 samples at higher concentration (5 mg/plate) were toxic to tester bacteria. With chlorine dioxide-tryptophan reaction mixtures, mutagenic activity was detected at all three ratios with 7:1 most potent (Table 2). Unlike the aqueous chlorine reaction products, no toxicity was observed in chlorine dioxide reaction mixtures even at doses up to 5 mg/plate (Table 2).

In general, addition of S9 mix to the assay system led to decline in mutagenicity. Binding of S9 to the test sample thus decreased the amount of test compound available for bacterial mutagenesis, or production of less toxic metabolites by the mixed function oxidase may have contributed to overall decrease in mutagenic activity. This decrease was consistent with other mutagenicity data on water chlorination reactions.

Amberlite XAD resin adsorption

Amberlite XAD polymeric resins have received much recent attention in toxicology because of their capacity to concentrate minute quantities of organic mutagens from large volumes of aqueous solutions (Vartiainen et al., 1987; Yamasaki and Ames, 1977). Amberlite XAD-2 resins have been used alone (Horth et al., 1987) or in combination with Amberlite XAD-8 (Ringhand et al., 1987). XAD-4 resins have also been used alone (Vartianen et al., 1987) or in combination with XAD-8 (Kool et al., 1981). The results of the Amberlite XAD-2/8 study are presented in Tables 3 and 4. In strain TA98, a dose-related increase in mutagenic activity was observed in acetone eluates of the aqueous chlorine-tryptophan reaction products. When samples were tested at 100μL/plate, the mutagenic activity increased with increasing chlorine concentration. The metha-

Table 1—Mutagenicity of ethyl ether extracts of aqueous chlorination of tryptophan[a]

		Number of revertants/plate[b]							
		TA98				TA100			
Sample	Dose/plate	−S9	MR[c]	+S9	MR	−S9	MR	+S9	MR
Bacteria		20 ± 6		29 ± 3		123 ± 9		120 ± 9	
Bacteria + DMSO	25 μL	21 ± 3		28 ± 4		124 ± 9		124 ± 5	
1:1 Extract	1 mg	42 ± 9	2.0	36 ± 6	1.3	106 ± 9	0.9	126 ± 27	1.0
	2 mg	58 ± 6	2.8	47 ± 10	1.7	156 ± 20	1.3	151 ± 9	1.2
	5 mg	68 ± 12	3.2	42 ± 11	1.5	134 ± 10	1.1	217 ± 23	1.8
3:1 Extract	250 μg	29 ± 5	1.4	36 ± 5	1.3	ND[d]		ND	
	1 mg	38 ± 2	1.8	47 ± 5	1.7	179 ± 37	1.4	143 ± 7	1.2
	2 mg	ND		ND		106 ± 21	0.9	146 ± 13	1.2
	5 mg	T[e]		T		T		T	
7:1 Extract	500 μg	174 ± 18	8.3	68 ± 7	2.4	ND		ND	
	1 mg	237 ± 16	11.3	92 ± 2	3.3	667 ± 51	5.4	407 ± 32	3.3
	2 mg	ND		ND		706 ± 71	5.7	644 ± 59	5.2
	5 mg	T		285 ± 8	10.2	T		1203 ± 123	9.7

[a] Three molar ratios of aqueous chlorine and tryptophan (1:1, 3:1 and 7:1) reacted at room temperature 24 hr. product from each reaction extracted separately with ethyl ether, concentrated and assayed for mutagenic activity in *Salmonella typhimurium* strains TA98 and TA100.
[b] Means and standard deviations of duplicate runs, each with 4 plates/dose.
[c] Mutagenicity ratio (MR) = number of revertants per test dose/controls.
[d] Not determined.
[e] Toxic.

Table 2—Mutagenicity of ethyl ether extracts of reactions of chlorine dioxide with tryptophan[a]

		Number of revertants/plate[b]							
		TA98				TA100			
Sample	Dose/plate	−S9	MR[c]	+S9	MR	−S9	MR	+S9	MR
Bacteria		25 ± 3		22 ± 4		135 ± 11		123 ± 3	
Bacteria + DMSO	25 μL	21 ± 3		22 ± 7		140 ± 14		118 ± 8	
1:1 Extract	1 mg	63 ± 9	3.0	30 ± 6	1.4	182 ± 13	1.3	159 ± 20	1.3
	2 mg	111 ± 6	5.3	64 ± 9	3.0	371 ± 42	2.7	216 ± 12	1.8
	5 mg	168 ± 12	8.0	152 ± 11	7.2	599 ± 19	2.7	497 ± 90	4.2
3:1 Extract	1 mg	34 ± 10	1.6	112 ± 4	5.3	176 ± 21	1.3	163 ± 18	1.4
	2 mg	126 ± 37	6.0	95 ± 11	4.5	342 ± 19	2.4	257 ± 23	2.1
	5 mg	112 ± 8	5.3	101 ± 10	4.8	659 ± 56	4.7	567 ± 55	4.8
7:1 Extract	1 mg	107 ± 16	5.1	57 ± 8	2.7	252 ± 13	1.8	244 ± 32	2.1
	2 mg	163 ± 25	7.8	98 ± 14	4.7	449 ± 64	3.2	330 ± 4	2.8
	5 mg	250 ± 33	11.9	141 ± 5	6.7	844 ± 67	6.0	605 ± 57	6.7

[a] Three molar ratios of chlorine dioxide and tryptophan (1:1, 3:1 and 7:1) reacted at room temperature 24 hr. Products from each reaction extracted separately with ethyl ether, concentrated and assayed for mutagenic activity in *Salmonella typhimurium* strains TA98 and TA100.
[b] Means and standard deviations of duplicate runs, each with 4 plates/dose.
[c] Mutagenicity ratio (MR) = number of revertants per test dose/controls.

Table 3—Mutagenicity of Amberlite XAD eluates of the reaction of aqueous chlorine with tryptophan[a]

		Number of revertants/plate[b]							
		TA98				TA100			
Sample	Dose/plate	−S9	MR[c]	+S9	MR	−S9	MR	+S9	MR
Bacteria		20 ± 6		29 ± 3		123 ± 9		120 ± 9	
Bacteria + DMSO	25 μL	21 ± 3		28 ± 4		124 ± 9		124 ± 5	
1:1 Acetone	100 μL	86 ± 25	4.3	52 ± 14	1.8	225 ± 14	0.9	126 ± 27	1.3
eluate	200 μL	196 ± 7	9.8	73 ± 8	2.5	299 ± 56	2.4	254 ± 4	2.1
	300 μL	T[d]		T		T		T	
1:1 Methanol	1 mg	38 ± 6	1.8	44 ± 7	1.6	141 ± 8	1.1	163 ± 21	1.3
eluate	2 mg	44 ± 2	2.1	36 ± 12	1.3	152 ± 16	1.2	164 ± 21	1.3
	5 mg	85 ± 12	4.0	56 ± 11	2.0	129 ± 19	1.0	173 ± 3	1.4
3:1 Acetone	50 μL	107 ± 20	5.4	39 ± 6	1.3	188 ± 30	1.5	85 ± 12	0.7
eluate	100 μL	138 ± 9	6.9	48 ± 4	1.7	214 ± 30	1.7	310 ± 29	2.6
	200 μL	T		T		T		T	
3:1 Methanol	1 mg	35 ± 2	1.7	45 ± 2	1.6	134 ± 3	1.1	135 ± 4	1.1
eluate	2 mg	60 ± 8	2.9	50 ± 8	1.8	156 ± 11	1.3	153 ± 15	1.2
7:1 Acetone	50 μL	199 ± 13	10.0	114 ± 8	3.9	1556 ± 151	12.7	785 ± 53	6.3
eluate	100 μL	313 ± 30	15.7	126 ± 28	4.3	2205 ± 55	17.9	989 ± 77	8.2
	200 μL	T		T		T		T	
Acetone eluate	500 μg	71 ± 8	3.4	46 ± 6	1.6	539 ± 34	4.3	459 ± 43	3.7
precipitate[e]	2 mg	137 ± 21	6.5	95 ± 10	3.4	491 ± 62	4.0	717 ± 60	6.0
	5 mg	T		T		T		T	
7:1 Methanol	500 μg	29 ± 4	1.4	50 ± 5	1.8	124 ± 11	1.0	151 ± 7	1.2
elute	1 mg	30 ± 6	1.4	55 ± 8	2.0	158 ± 7	1.3	177 ± 8	1.4

[a] Three molar ratios of aqueous chlorine and tryptophan (1:1, 3:1 and 7:1) reacted at room temperature 24 hr. The products from each reaction were concentrated separately by Amberlite XAD adsorption. The adsorbed compounds were eluted separately with acetone and then methanol, concentrated and assayed for mutagenic activity in *Salmonella typhimurium* strains TA98 and TA100.
[b] Means and standard deviations of duplicate runs, each with 4 plates/dose.
[c] Mutagenicity ratio (MR) = number of revertants per test dose/controls.
[d] Toxic.
[e] A precipitate formed after removing acetone, dissolved in DMSO, assayed for mutagenic activity.

Table 4—Mutagenicity of Amberlite XAD eluates of the reaction of chlorine dioxide with tryptophan[a]

Sample	Dose/plate	Number of revertants/plate[b] TA98 −S9	MR[c]	TA98 +S9	MR	TA100 −S9	MR	TA100 +S9	MR
Bacteria		25 ± 3		23 ± 3		135 ± 11		123 ± 3	
Bacteria + DMSO	25 μL	21 ± 3		21 ± 3		140 ± 10		118 ± 8	
1:1 Acetone	100 μL	121 ± 22	4.8	70 ± 6	3.0	332 ± 18	2.7	240 ± 14	2.0
eluate	200 μL	177 ± 33	7.1	62 ± 16	2.7	391 ± 29	2.9	400 ± 16	3.3
	300 μL	T[d]		T		T		T	
1:1 Methanol	1 mg	41 ± 3	1.9	38 ± 5	1.9	152 ± 12	1.1	142 ± 17	1.2
eluate	2 mg	35 ± 14	1.7	40 ± 6	1.9	152 ± 12	1.1	145 ± 8	1.2
3:1 Acetone	50 μL	74 ± 3	3.0	43 ± 4	1.9	ND[e]		ND	
eluate	100 μL	112 ± 13	4.5	58 ± 4	2.5	463 ± 53	3.4	496 ± 28	4.0
	200 μL	97 ± 27	3.9	62 ± 8	2.7	529 ± 44	3.9	512 ± 71	4.2
3:1 Methanol	1 mg	26 ± 11	1.2	46 ± 6	2.3	148 ± 12	1.1	122 ± 4	1.0
eluate	2 mg	31 ± 15	1.5	56 ± 11	2.8	158 ± 13	1.1	122 ± 11	1.0
7:1 Acetone	100 μl	178 ± 32	7.1	98 ± 6	4.3	371 ± 67	2.7	131 ± 37	1.1
eluate	200 μL	213 ± 15	8.5	93 ± 16	4.0	373 ± 23	2.8	512 ± 37	4.2
	300 μL	161 ± 26	6.4	118 ± 12	5.1	656 ± 69	4.9	1036 ± 22	8.4
7:1 Methanol	1 mg	25 ± 4	1.2	32 ± 5	1.6	160 ± 28	1.1	113 ± 37	1.0
eluate	2 mg	30 ± 8	1.4	34 ± 7	1.7	167 ± 14	1.2	131 ± 11	1.0

[a] Three molar ratios of chlorine dioxide and tryptophan (1:1, 3:1 and 7:1) reacted at room temperature 24 hr. The products from each reaction were concentrated separately by Amberlite XAD adsorption. The adsorbed compounds were eluted separtely with acetone and then methanol, concentrated and assayed for mutagenic activity in *Salmonella typhimurium* strains TA98 and TA100.

[b] Means and standard deviations of duplicate runs, each with 4 plates/dose.

[c] Mutagenicity ratio (MR) equals number of revertants per test dose/controls.

[d] Toxic.

[e] Not determined.

Table 5—Percent distribution of radioactivity after resin adsorption of filtrates of aqueous reaction mixtures of chlorine or chlorine dioxide with tryptophan[a]

Sample fraction	Tryptophan + HOCl (1:1 ratio)	Tryptophan + ClO_2 (1:1 ratio)	Tryptophan + HOCl (7:1 ratio)	Tryptophan + ClO_2 (7:1 ratio)
Filtrate[b]	100	100	100	100
Column effluent	35.5 ± 2.9	36.1 ± 0.5	48.6 ± 2.0	41.2 ± 0.8
Column eluate[c]	56.5 ± 3.1[x]	60.6 ± 1.8[x]	49.5 ± 0.7[y]	48.6 ± 1.2[y]
Loss[d]	7.8	3.3	3.7	10.2

[a] Aqueous chlorine or chlorine dioxide reacted with radiolabelled tryptophan (1:1 and 7:1 molar ratio) at room temperature 24 hr. Excess chlorine or chlorine dioxide removed by addition of sodium sulfite, solutions filtered and concentrated by resin adsorption; adsorbed substances eluted with acetone and methanol, eluates combined, and concentrated by rotary evaporation.

[b] Filtrate assumed 100% radioactivity. After chlorination of tryptophan, there was about 20% loss of initial radioactivity.

[c] Means and standard deviations from duplicate runs of four samples each. Means with same letter in same row not significantly different.

[d] Radioactivity not accounted for assumed trapped in resins or volatilized.

nol eluates were weakly mutagenic at 1:1 and 3:1 but not at 7:1 (Table 3).

In strain TA100, high mutagenic activity was observed for the acetone eluate at 7:1. Addition of S9 mix caused reduction of the activity. The methanol eluates were not mutagenic to strain TA100. These results are in agreement with other published data (Horth et al., 1987; Tan et al., 1987). Horth et al. (1987) and Tan et al. (1987) used Amberlite XAD concentration procedures to evaluate mutagen formation using equimolar concentrations of tryptophan and aqueous chlorine, and observed mutagenic activity in concentrates. In our study, the reactants were mixed at different molar ratios, and mutagenic activity was detected. A precipitate formed upon removing acetone and dissolved in DMSO was found to be mutagenic to both strains (Table 3).

The acetone eluates of all chlorine dioxide products were mutagenic to both strains either with or without metabolic activation (Table 4). The methanol eluates were either nonmutagenic or weakly mutagenic. Tan et al. (1987) reported the mutagenic activity of chlorine dioxide-tryptophan reaction products. Their results indicated the reaction products of chlorine dioxide and tryptophan were not mutagenic at 1:1 molar ratio. Discrepancies between that and our work may be due to their use of only Amberlite XAD-2 to concentrate reaction products at pH 6.0 followed by ethyl ether elution; they recovered less than 12% of the total applied radioactivity. In our study, the use of both Amberlite XAD-2 and XAD-8 in one column with sample acidification to pH 2.5 allowed better recovery (about 60%) of reaction products in the filtrate of the 1:1 mixtures (Table 5). Recovery of applied radioactivity in the 7:1 mixture was significantly less than in the 1:1 ratio for both aqueous chlorine and chlorine dioxide (Table 5).

About 20% of the original radioactivity was in the precipitate or lost through volatilization. This was not surprising since tryptophan has been reported as a prolific producer of chloroform (Morris and Baum, 1978). According to Morris and Baum (1978), the molar chloroform production from reaction of chlorine and tryptophan approached 100% at higher pH. Losses were observed after resin adsorption, which could be the result of volatilization or inability to desorb radioactive products from resins.

TLC fractionation of products

The highly mutagenic acetone eluate from the XAD 2/8 column of aqueous chlorine reaction mixture at 7:1 was further fractionated by TLC. Five fluorescent bands (designated #1 to 5) with varying intensity were detected under UV light at 366 nm. Bands 2 and 5 showed blue fluorescence and the other three green. The chlorine dioxide reaction produced four fluorescent bands (designated A to D); bands A and B green, and bands C and D blue.

Compounds present in bands 4 and 5 were highly mutagenic toward TA98 and TA100 (Table 6). Those in band 2 in absence of S9 were also highly mutagenic toward TA98. The mutagenic activity was greatly reduced when the S9 mix was included.

Fractioned samples from chlorine dioxide mixtures were either weakly mutagenic or non-mutagenic toward either tester strain (Table 7). Addition of S9 did not increase activity.

Fluorescent compounds have previously been identified from reactions of alkaline hypochlorite with certain tryptophan derivatives (van Temelen et al., 1968). Ohara et al. (1988) detected several colored products under UV from reactions of tryptophan and nitrite under acidic conditions. However, ours is the first report in which several fluorescent compounds were

Table 6—Mutagenicity of TLC fractions of Amberlite XAD eluate of aqueous chlorine-tryptophan reaction products[a]

		Number of revertants/plate[b]							
		TA98				TA100			
Sample	Dose/plate	−S9	MR[c]	+S9	MR	−S9	MR	+S9	MR
Bacteria		27 ± 2		28 ± 7		120 ± 13		89 ± 14	
Bacteria + DMSO	25 μL	22 ± 2		26 ± 7		116 ± 6		95 ± 2	
Band 1 R_f = 0	250 μg	22 ± 4	1.0	31 ± 7	1.2	113 ± 4	1.0	111 ± 9	1.2
	1 mg	33 ± 5	1.5	28 ± 2	1.1	158 ± 15	1.4	110 ± 14	1.2
	5 mg	78 ± 6	2.8	30 ± 6	1.2	321 ± 30	2.8	124 ± 20	1.3
Band 2 R_f=0.08	250 mg	44 ± 6	2.0	30 ± 7	1.2	225 ± 15	1.9	113 ± 18	1.2
	1 mg	174 ± 15	7.9	36 ± 5	1.4	594 ± 72	5.1	144 ± 16	1.7
	5 mg	T[d]		56 ± 10	2.2	3987 ± 186	34.4	430 ± 39	4.5
Band 3 R_f=0.22	250 μg	26 ± 4	1.2	28 ± 6	1.1	180 ± 19	1.6	126 ± 9	1.3
	1 mg	118 ± 10	5.4	34 ± 5	1.3	915 ± 22	7.9	166 ± 6	1.7
	5 mg	T		71 ± 19	2.7	T		322 ± 33	5.5
Band 4 R_f=0.38	250 μg	62 ± 10	2.8	26 ± 6	1.0	267 ± 26	2.3	130 ± 15	1.4
	1 mg	38 ± 8	6.3	28 ± 10	1.1	1420 ± 88	12.2	154 ± 11	1.6
	5 mg	598 ± 59	27.2	62 ± 8	2.4	T		348 ± 113	6.8
Band 5 R_f=0.54	250 μg	53 ± 6	2.4	30 ± 4	1.2	177 ± 20	1.5	146 ± 15	1.2
	1 mg	111 ± 8	5.0	44 ± 5	1.7	1017 ± 96	8.8	213 ± 20	2.2
	5 mg	418 ± 20	19.0	89 ± 7	3.4	4128 ± 419	35.6	728 ± 47	7.6

[a] Aqueous chlorine and tryptophan (7:1 ratio) reacted at room temperature 24 hr. Excess chlorine removed by sodium sulfite, products concentrated by Amberlite XAD adsorption, eluted with acetone, fractionated by TLC on silica gel G with hexane:ethyl ether: acetic acid (50:50:2, v/v). Bands from plates scraped, extracted and assayed.
[b] Means and standard deviations of duplicate runs, each with 4 plates/dose.
[c] Mutagenicity ratio (MR) = number of revertants per test dose/controls.
[d] *Toxic.*

Table 7—Mutagenicity of TLC fractions of Amberlite XAD eluate of reaction products of chlorine dioxide with tryptophan[a]

		Number of revertants/plate[b]							
		TA98				TA100			
Sample	Dose/plate	−S9	MR[c]	+S9	MR	−S9	MR	+S9	MR
Bacteria		30 ± 7		29 ± 5		106 ± 12		109 ± 8	
Bacteria + DMSO	25 μL	28 ± 9		26 ± 7		98 ± 7		94 ± 7	
Band A R_f=0	250 μg	47 ± 6	1.7	26 ± 2	1.0	129 ± 18	1.3	122 ± 9	1.3
	1 mg	51 ± 5	1.8	33 ± 5	1.3	118 ± 22	1.2	118 ± 10	1.3
	5 mg	79 ± 14	2.8	29 ± 1	1.1	149 ± 18	1.5	129 ± 9	1.4
Band B R_f=0.13	250 μg	41 ± 2	1.5	27 ± 9	1.0	128 ± 25	1.3	122 ± 9	1.3
	1 mg	56 ± 2	2.0	33 ± 5	1.3	169 ± 23	1.7	158 ± 16	1.7
	5 mg	84 ± 3	3.2	33 ± 2	1.3	275 ± 23	2.8	214 ± 34	2.5
Band C R_f=0.31	250 μg	ND[d]		ND		ND		ND	
	1 mg	83 ± 4	3.0	33 ± 4	1.3	281 ± 35	2.9	250 ± 13	2.7
	5 mg	132 ± 15	4.7	60 ± 6	2.3	532 ± 65	5.4	489 ± 12	5.2
Band D R_f=0.44	250 μg	40 ± 5	1.4	27 ± 4	1.0	103 ± 15	1.1	109 ± 10	1.2
	1 mg	49 ± 5	1.8	32 ± 4	1.2	123 ± 14	1.3	106 ± 10	1.1
	5 mg	74 ± 7	2.6	28 ± 1	1.1	147 ± 15	1.5	128 ± 17	1.4

[a] Chlorine dioxide and trypophan (7:1 ratio) reacted at room temperature 24 hr. Excess chlorine removed by sodium sulfite, products concentrated by Amberlite XAD adsorption, eluted with acetone, concentrated and fractionated by TLC on silica gel G plates with hexane:ethyl ether:methanol:acetic acid (70:30:7:2, v/v). Bands from the TLC plates scraped, extracted and assayed.
[b] Means and standard deviation of duplicate runs, each with 4 plates/dose.
[c] Mutagenicity ratio (MR) = number of revertants per test dose/controls.
[d] Not Determined.

Table 8—Distribution of radioactivity in TLC fractions of reaction products of aqueous chlorine or chlorine dioxide with tryptophan[a]

Aqueous chlorine	% Radioactivity[b]	Chlorine dioxide	% Radioactivity
Band #		Band #	
1 (R_f=0)	64.1	A (R_f=0)	15.0
2 (R_f=0.08)	11.0	B (R_f=0.13)	77.0
3 (R_f=0.22)	8.7	C (R_f=0.31)	2.0
4 (R_f=0.38)	1.8	D (R_f=0.44)	0.7
5 (R_f=0.54)	1.4		
Gaps[c]	3.0	Gaps	0.3

[a] Tryptophan solutions in 0.1 M soduim phosphate buffer was added radioactive tryptophan of known activity, with aqueous chlorine or chlorine dioxide at 1:7 molar ratio 24 hr. Excess chlorine or chlorine dioxide removed with sodium sulfite, filtered and concentrated by Amberlite XAD adsorption, eluted with acetone, concentrated, and fractionated on silica gel G plates, fractions scraped and radicactivity checked.
[b] Percent radioactivity determined by dividing the radioactivity count of each band by the total radioactivity applied.
[c] Silica gel between bands also scraped off and checked.

produced from reactions of aqueous chlorine or chlorine dioxide with tryptophan.

Analysis of the TLC bands for radioactivity indicated most radioactivity was in the polar fractions which accounted for very little mutagenic activity (Table 8). The highly mutagenic fraction (Band #5) of the aqueous chlorine products accounted for about 1.4% of the radioactivity, while that of the chlorine dioxide products (Band #C) accounted for only 2% (Table 8). These results were expected since very minute quantities of organic mutagens are produced in chlorine treated drinking water (Tardiff et al., 1978).

Identification of the reaction products

Due to the high mutagenic activity in band #5 of the TLC fraction of the aqueous chlorine-tryptophan reaction it was further examined using GC/MS. Three chlorinated compounds eluting at 9.03, 10.15, and 14.9 min were identified as 1,1,3-trichloropropanone, 1,1,3,3-tetrachloropropanone and 1,2-dichloroquinoline based on molecular weights from chemical ionization (CI) spectra and numbers of chlorine atoms from isotope cluster patterns in both CI and electron impact (EI) spectra (data not shown). 1,1,3,3-Tetrachloropropanone and 1,2-dichloroquinoline were also identified by computer match-

ing of the EI sample spectra with an on-line library of 42,223 published EI spectra (EPA/NIH Mass Spectra Data Base, NISTM, Washington, DC). In both cases the designated compound provided the closest match. Computer matching was not possible for 1,1,3-trichloropropanone because the spectrum of that compound was not in the library. However, the identification was supported by molecular weight and isotope cluster data and by the fact that fragment ions in the experimental EI spectrum were analogous to the library spectrum of 1,1,1-trichloropropanone.

The chlorinated ketone derivatives have previously been identified from chlorinated humic acid solutions and reported to be mutagenic to TA100 without metabolic activation (Coleman et al., 1983; Kringstad et al., 1983; Meier et al., 1986). 1,1,3,3-Tetrachloropropanone was also reported to be a precursor of a potent bacterial mutagen, 3-chloro-4-(dichloromethyl)-5-hydroxy-2-(5H)-furanone (MX, Padmapriya et al., 1985). Both compounds are known precursors of the haloform reaction and their formation from tryptophan may explain the production of chloroform (Morris and Baum, 1978). The mechanism to form the haloacetone derivatives is unknown but thought to involve ring chlorination and subsequent opening followed by further chlorination (Rook, 1980). Tryptophan and some derivatives reported to react with hypohalites to produce quinoline-type compounds (van Temelen et al., 1968) may explain the formation of dichloroquinoline.

Effectiveness of liquid-liquid extraction and Amberlite XAD adsorption in concentrating mutagens

We compared effectiveness of LLE and XAD adsorption for concentrating mutagens from chlorinated tryptophan reaction mixtures at these three molar ratios (Tables 1–4). Based on total mutagenic activity, XAD resin adsorption was superior to LLE for concentrating organic mutagens. The number of revertants in LLE samples at 1 mg/plate was much lower than that of acetone eluates of the XAD adsorbed samples at 0.1 mL/plate. LLE has been used by several researchers for mutagenicity studies (Grabow et al., 1981; Vartiainen et al., 1987) or chemical analysis (Trehy et al., 1986). Vartiainen and colleagues (1987) reported LLE by magnetic mixing was inefficient in concentrating mutagens from drinking water. However, continuous LLE was comparable to Amberlite XAD resin adsorption, especially when longer extraction times were used (Vartiainen et al., 1987).

Note that because reaction products in the aqueous chlorine-tryptophan and chlorine dioxide-tryptophan mixtures are different in chemistry and quantity, they were not expected to perform similarly in the two concentration systems. In addition, the presence of any anti-mutagenic or co-mutagenic compounds would also affect the total activity of the mixtures. Comparison of efficiency in concentrating mutagenic reaction products can thus be performed only on the basis of total mutagenic activity.

CONCLUSION

Mutagens were clearly produced when aqueous chlorine or chlorine dioxide reacted with tryptophan. Based on total mutagenic activity, it was very difficult to predict which chlorinating agent was better unless the mutagenic compounds produced in the reaction mixtures were identified and quantities determined. Rapson et al. (1985) reported mutagenic activity of chlorinated tyrosine solution decreased when chlorine was replaced with chlorine dioxide. Our study did not clearly indicate that even though different amino acids were used. The considered replacement of chlorine with chlorine dioxide for water treatment (USEPA, 1983) should be evaluated further in light of our results because even though chlorine dioxide did not produce THMs, it was reported to produce mutagens when used for drinking water disinfection (de Greef et al., 1980; Kool et al., 1985).

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Interactions Among Calcium, Zinc and Phytate with Three Protein Sources

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ABSTRACT

Various combinations of calcium (4.94 mmol), zinc (0.0071 mmol) and phytate (0.284 mmol) were added either to soy concentrate, casein or torula yeast to determine effects of their interaction on *in vitro* solubility of protein, calcium, zinc, and phytate (PA). Two Ca sources, calcium carbonate ($CaCO_3$) and calcium-citrate-malate complex (CCM) were used. Two pH levels, 2.0 and 5.5, were used to simulate gastrointestinal pH conditions. An increase in pH significantly reduced ($P<0.01$) Zn solubility in all treatments with all protein sources. The solubility of Ca and PA were significantly decreased ($P<0.01$) when both components were present probably due to formation of insoluble Ca-PA complexes. At pH 5.5, with casein and yeast proteins, Zn was significantly more ($P<0.01$) soluble in samples with CCM, in the absence of PA, than in those with $CaCO_3$.

INTRODUCTION

There is growing consumer concern over the nutritional adequacy of the human diet. Recently, public awareness of calcium need has increased with linkage of osteoporosis to inadequate calcium intake. As a result many foods are being fortified with calcium. However, interactions among nutrients can affect the absorption, metabolism and/or excretion of other nutrients and it is therefore important to determine how calcium interacts with other food components. Studies with animals indicated that calcium interfers with absorption of zinc when phytate is present (Forbes et al., 1984; Beal et al., 1984). Mills (1985) suggested that the calcium content determined zinc availability in phytate rich diets as calcium has a synergistic effect on the phytate-zinc antagonism and the mmolar ratio of PA X Ca:Zn in a diet has been suggested as a predictor of zinc bioavailability (Fordyce et al., 1987; Ellis et al, 1987; Bindra et al., 1986). The results of recent studies (Bindra et al., 1986; Ellis et al., 1987) indicated that humans that have a PA X Ca:Zn mmolar ratio above 200 may have increased risk of impaired zinc bioavailability. Protein has also been shown to affect mineral absorption.

A nutrient, to be bioavailable, needs to be in soluble form; however, not all soluble complexes are bioavailable. The purpose of our research was to gain a better understanding of the solubility characteristics of various constituents which might take part in such complexes. Calcium, zinc and phytate in various combinations were added to one of three protein sources: soy concentrate, casein and torula yeast, to form a mmolar concentration of approximately 200 mmolar. Two different calcium sources were examined. Due to the recent interest in CCM (calcium-nitrate-malate complex) as a possible calcium fortificant or supplement (Smith et al., 1987; Smith 1988) it was evaluated versus $CaCO_3$ (calcium carbonate). Two pH levels, 2.0 and 5.5, were used to simulate gastrointestinal pH conditions. Total and soluble phytate and protein were determined in each system.

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MATERIALS & METHODS

Materials

Soy protein concentrate, Procon ® Plus, casein and torula yeast were obtained courtesy of Procter and Gamble Company (Ross, OH).

Reagents

All reagents were analytical grade and solutions prepared with double distilled deionized water (DDW). All glassware was acid washed in concentrated HCl and rinsed with DDW.

Calcium carbonate and zinc oxide were obtained from Fisher Scientific, calcium-citrate-malate complex (CCM) was obtained courtsey of Procter and Gamble and sodium inositol hexaphosphoric acid dodecasodium salt was obtained from Sigma Chemical Company Company (St. Louis, MO).

Mineral and phytate stock solutions were formulated from calcium carbonate (1102 mM), CCM (830 mM), zinc oxide (1.3 mM) and dodecasodium phytate (142 mM) such that the concentrations of calcium (4.94 mmol), zinc (0.0071 mmol) and phytate (0.0284 mmol) provided a phytate X calcium:zinc mmolar ratio of approximately 200 mmolar when all the components were present in a treatment as shown in Table 1.

Apparatus

A Perkin-Elmer model 2380 atomic absorption spectrophotometer (AAS) with an air acetylene flame, single slot head and Pt-Rh corrision resistant nebulizer was used to measure calcium concentration at 422.7 nm and zinc at 213.9 nm. A Perkin-Elmer Lambda 3 UV/VIS Spectrophotometer was used for absorption measurements.

Experimental design

Various combinations of calcium, zinc and phytate were added to the protein sources to determine the interaction among components and to determine the effects of these interactions on the solubility of protein, phytate, calcium, and zinc. These are listed in Table 2. In this way it was possible to determine which component(s) caused the formation of insoluble complexes and the extent of the insolubility. Each protein source was added to each system described in Table 2.

Sample preparation

The appropriate amounts of calcium and/or zinc and/or phytate were added from stock solutions to DDW at pH 2.0 so the final volume was 100 mL. All stock solutions and the DDW were acidified to pH

Table 1—Concentrations (mmoles) of two sources of calcium, zinc and phytate added to three protein sources: casein, soy concentrate and torula yeast and their respective PA X Ca:Zn mmolar ratios

Treatment[a]	Phytate (mmol)	Calcium (mmol)	Zinc (mmol)	PA × Ca:Zn (mmolar)
Casein				
$CaCO_3$	0.284	5.02	0.0071	200.80
CCM	0.247	5.26	0.0071	183.00
Soy				
$CaCO_3$	0.256	5.14	0.0071	189.40
CCM	0.247	5.26	0.0071	183.00
Yeast				
$CaCO_3$	0.255	4.97	0.0071	185.71
CCM	0.282	5.26	0.0071	208.92

[a] $CaCO_3$ = Calcium carbonate; CCM = Calcium-citrate-malate

Table 2—Combinations of calcium, zinc and phytate added to casein, soy concentrate and torula yeast protein sources[a]

System	Components added Calcium (5.14mmol)	Zinc (0.0071 mmol)	Phytate (0.282 mmol)
1	+	−	−
2	−	−	+
3	−	+	−
4	+	−	+
5	−	+	+
6	+	+	+
7	−	−	−
8	+	+	−

[a] The addition of minerals at the concentration indicated is shown by the symbol +. If not added the symbol − is shown.

2.0 with 6.0N and 1.0N HCl, so that no precipitation of minerals or phytate would occur before the addition of the protein source. The calcium stock solution (if present) was added first to the DDW, followed by the zinc (if present) and then phytate (if present). This order of addition of the parameters was followed for all experiments as order can affect binding (Nosworthy and Caldwell, 1988; Platt and Clydesdale, 1983). The minerals, phytate and DDW were allowed to equilibrate stirring constantly at room temperature (20°C) by far 20 min. The protein source (calculated from total protein and moisture to provide 1.555g protein) was added and the mixture stirred at medium speed an additional 30 min at 20°C. The pH of the slurry was recorded and the pH was reduced to pH 2.0±0.05 with 6.0N and 1.0N HCl. The mixture was stirred an additional 30 min at 20°C and samples were removed for mineral, protein, and phytate analysis. The pH of the slurry was then adjusted to pH 5.5±0.05 with 6.0N, 1.0N, 0.1N, and 0.01N NaOH, and stirred for 30 min at 20°C and samples were again removed for mineral, protein, and phytate analysis.

Analysis of mineral

Standards of 5.00 ppm calcium and 1.00 ppm zinc were prepared from 1000 ppm calcium and zinc Fisher Certified atomic absorption reference solutions.

Total minerals

A 10 mL aliquot of each sample was pipetted directly from the slurry into 100 mL digestion flasks. Twenty ml concentrated HCl and two glass beads were added to each flask. The samples were covered with Parafilm and left overnight (almost 20 hr) at 20°C. The samples were digested on a micro-Kjeldahl digestor for 30 min on medium heat. Upon cooling the samples were filtered through Whatman 40 ashless filter paper into 100 ml volumetric flasks. The flasks were made up to volume with DDW and in most cases this sample served for zinc analysis by AAS. A 1 mL aliquot was taken from the 100 mL volumetric and pipetted into a 50 mL volumetric flask which contained 5 mL of lanthanum chloride. The flask was then made up to volume with DDW and this sample analyzed for calcium by AAS. Lanthanum chloride solution was added so that its final concentration was 0.5%.

Soluble minerals

A 45 mL aliquot of each sample was centrifuged at a relative centrifugal force of 3335 X *g* for 20 min. A 10 mL aliquot of the supernatant was pipetted into a 100 ml digestion flask and 20 mL of concentrated HCl and two glass beads were added. The remainder of the analysis procedure was the same as for total mineral determination described above.

Total and soluble protein and phytate determination

Total protein was determined using a modified AOAC (1980) method. Soluble protein in the supernatant was measured by the method of Lowry et al., (1951) as adapted by Petersen (1977) using protein assay kits (Sigma Chemical Co.). Phytate was determined on 1 ml aliquot of supernatant using a modification of the ion exchange procedure developed by Ellis and Morris (1983, 1986). Phosphorus was determined by the method of Fiske and Subbarow (1925).

Calcium carbonate ($CaCO_3$) versus calcium-citrate-malate (CCM)

A CCM stock solution was formulated to provide similar calcium concentration to that of the $CaCO_3$ studies. CCM replaced $CaCO_3$ in all treatments for each of the three protein sources. The experimental procedure was identical to that followed when $CaCO_3$ was added.

Statistical analysis

All treatments were conducted in at least triplicate. The biomedical computer program BMDP&D (Health Sciences Computer Facilities, Univ. of California, Los Angeles) adapted to the Univ. of Massachusetts computer center was used to perform two-way analysis of variance using the Scheffe test for significance (Winer, 1962). Significance was noted at $P<0.05$ and $P<0.01$.

RESULTS & DISCUSSION

THE ENDOGENOUS LEVELS of calcium, zinc, phytate and total protein and moisture in the three protein sources are listed in Table 3.

Soluble protein

At pH 2.0 the addition of both calcium ($CaCO_3$ and CCM) and phytate (PA) or a combination of the two significantly reduced ($P<0.01$) soluble protein in both soy concentrate and casein except in soy concentrate with calcium (Pr (protein) + Ca) where the reduction was significant at the 5% level (Table 4). With the yeast protein, treatments containing CCM had significantly more ($P<0.01$) soluble protein than any treatment containing combinations of zinc and phytate but were not significantly different from treatments containing $CaCO_3$. We noted that yeast protein has considerably more endogenous zinc than the other two protein sources so the extra cations provided by that zinc may be a factor in explaining the calcium effect on protein solubility.

Grynspan and Cheryan (1989) found calcium did not alter soy protein solubility at a pH below the isoelectric point, however, the calcium concentrations they tested were considerably lower than those we used.

The observation that soluble protein decreased when phytate was added to soy concentrate or casein agrees with research reported by Grynspan and Cheryan (1989) who found the solubility of protein and phytate decreased at low pH. Phytate binding below the isoelectric point of proteins is known to be a result of the strong electrostatic interaction between catonic residues on the protein (lysly, histdyl, arginyl and amino terminal groups) and the anionic phosphate ester of phytate. However, phytate added to yeast did not alter protein solubility. The addition of zinc did not alter protein solubility in any of the protein sources. However, when combined with calcium and/or phytate highly significant reductions at the 1% level occurred with soy concentrate and casein except with soy protein with CCM, Zn and PA ($P<0.05$). Yeast systems containing zinc and calcium had a significant increase ($P<0.01$) in protein solubility. However, the zinc added in these experiments was 0.0071 mmol and its effect may have been overwhelmed by much higher concentrations of calcium (5.0 mmol) or phytate (0.287 mmol) especially in the soy concentrate and casein where endogenous zinc was low.

An increase in pH from 2.0 to 5.5 altered protein solubility in the casein and soy concentrate (Table 4). The solubility of yeast protein was unaffected by the change. Protein solubility was significantly reduced ($P<0.01$) alone and with zinc, and a significant decrease ($P<0.05$) occurred in the Pr + Ca + Zn treatment with soy concentrate, most likely due to the conformational changes brought about by the passage of the protein through the isoelectric point (Table 4).

A significant increase ($P<0.05$) in soluble protein was ob-

Table 3—Endogenous calcium, zinc, phytate, total protein and moisture composition in casein, soy concentrate and yeast protein

Protein Source	Moisture (%)	Total protein (%)	Calcium (mg/g protein)	Zinc (μg/g protein)	Phytate (mg/g protein)
Casein	6.28 ± 0.01	97.19 ± 0.71	0.21 ± 0.01	36.81 ± 1.13	ND[a]
Soy	5.51 ± 0.04	68.47 ± 0.82	5.22 ± 0.09	41.73 ± 2.27	ND
Yeast	7.18 ± 0.04	49.48 ± 0.51	8.60 ± 0.19	385.45 ± 6.15	ND

[a] ND = Not Detected

Table 4—Means and standard deviations of soluble protein from three different protein sources, casein, soy concentrate and torula yeast, with and without added calcium, zinc and phytate at pH 2.0 and 5.5

	Soluble protein (mg)[a]		
	Protein sources[c]		
Treatments[b]	Casein	Soy concentrate	Yeast
pH 2.0			
Pr	707.29 ± 43.5a	146.92 ± 19.5a	55.50 ± 8.9cd
Pr + PA	0.00 ± 0.0b	26.63 ± 3.8cd	47.50 ± 0.0d
Pr + Ca	31.20 ± 1.1b	61.32 ± 10.9bc	87.50 ± 3.5abc
Pr + Ca + PA	2.07 ± 0.3b	63.19 ± 6.5b	75.50 ± 3.3abcd
Pr + Zn	733.39 ± 53.7a	136.36 ± 8.7a	56.67 ± 3.8bcd
Pr + Zn + PA	0.00 ± 0.0b	21.56 ± 5.2d	51.67 ± 5.2cd
Pr + Ca + Zn	28.38 ± 1.1b	92.00 ± 14.7b	101.00 ± 3.8a
Pr + Ca + Zn + PA	1.85 ± 0.2b	64.30 ± 6.1b	81.00 ± 2.6abcd
Pr + CCM	28.19 ± 1.3b	85.42 ± 24.3b	93.33 ± 1.4a
Pr + CCM + PA	1.33 ± 0.1b	65.40 ± 4.6b	84.58 ± 9.9ab
Pr + CCM + Zn	27.44 ± 1.2b	93.12 ± 19.1b	92.08 ± 6.9a
Pr + CCM + Zn + PA	0.50 ± 0.3b	60.88 ± 6.4bc	83.75 ± 7.5ab
pH 5.5			
Pr	301.10 ± 46.3a	31.31 ± 10.4a	65.60 ± 0.0a
Pr + PA	84.24 ± 23.9b	57.53 ± 5.5a	66.90 ± 1.8a
Pr + Ca	66.00 ± 1.3b	57.12 ± 8.5a	93.45 ± 10.7a
Pr + Ca + PA	27.23 ± 4.1d	46.99 ± 5.1a	80.80 ± 2.5a
Pr + Zn	365.32 ± 12.1a	49.32 ± 6.7a	75.75 ± 4.4a
Pr + Zn + PA	69.18 ± 19.5b	53.09 ± 4.2a	69.03 ± 1.4a
Pr + Ca + Zn	58.60 ± 4.2b	58.69 ± 5.4a	81.64 ± 5.2a
Pr + Ca + Zn + PA	24.66 ± 1.6d	48.10 ± 3.9a	72.37 ± 2.9a
Pr + CCM	67.58 ± 12.6b	57.72 ± 5.6a	89.25 ± 2.5a
Pr + CCM + PA	28.62 ± 3.5d	55.84 ± 5.0a	82.93 ± 10.9a
Pr + CCM + Zn	80.94 ± 2.4bc	52.15 ± 6.1a	85.42 ± 3.5a
Pr + CCM + Zn + PA	36.34 ± 6.2cd	48.83 ± 2.6a	81.60 ± 7.0a

[a] Means and standard deviations for a minimum of three replicates.
[b] Pr = protein, Ca = $CaCO_3$, CCM = calcium-citrate-malate complex, Zn = zinc and PA = phytate.
[c] Means within the same columns and pH level with different lower case letters are significantly different at $p < 0.05$.

Table 5—Means and standard deviations of soluble calcium from three different protein sources, casein, soy concentrate and torula yeast, with and without added zinc and phytate at pH 2.0 and pH 5.5

	Soluble calcium (mg/g protein)[a]		
	Protein sources[c]		
Treatments[b]	Casein	Soy concentrate	Yeast
pH 2.0			
Pr	0.22 ± 0.0c	3.47 ± 0.1b	8.36 ± 0.0b
Pr + PA	13.45 ± 0.4b	3.20 ± 0.0b	6.86 ± 0.5b
Pr + Ca	130.52 ± 1.4a	133.58 ± 0.2a	137.52 ± 7.4a
Pr + Ca + PA	129.32 ± 1.8a	128.59 ± 1.1a	125.70 ± 1.0a
Pr + Ca + Zn	126.55 ± 4.5a	135.90 ± 1.0a	123.87 ± 1.1a
Pr + Ca + Zn + PA	126.66 ± 2.9a	131.00 ± 1.3a	128.38 ± 2.1a
Pr + CCM	136.63 ± 2.3a	135.99 ± 3.7a	135.99 ± 0.9a
Pr + CCM + PA	130.41 ± 2.3a	133.25 ± 2.9a	132.94 ± 1.1a
Pr + CCM + Zn	135.83 ± 2.0a	136.47 ± 2.5a	125.22 ± 0.2a
Pr + CCM + Zn + PA	135.83 ± 1.4a	128.80 ± 2.3a	131.97 ± 1.6a
Total Ca	128.84 ± 3.5a	132.92 ± 13.5a	133.74 ± 2.3a
Total CCM	133.01 ± 4.3a	133.29 ± 3.4a	133.75 ± 3.8a
pH 5.5			
Pr	Not determined	1.82 ± 0.1c	5.63 ± 0.1c
Pr + PA	13.20 ± 0.8c	2.42 ± 0.1c	6.17 ± 1.1c
Pr + Ca	117.38 ± 0.8a	117.43 ± 2.8a	119.27 ± 2.4a
Pr + Ca + PA	81.39 ± 3.4b	78.56 ± 0.9b	87.34 ± 1.5b
Pr + Ca + Zn	117.99 ± 4.4a	116.46 ± 1.1a	114.45 ± 3.6a
Pr + Ca + Zn + PA	82.72 ± 5.9b	79.33 ± 2.9b	85.61 ± 2.0b
Pr + CCM	130.68 ± 11.4a	125.10 ± 10.5a	131.50 ± 0.9a
Pr + CCM + PA	84.11 ± 3.0b	81.49 ± 1.6b	89.19 ± 0.5b
Pr + CCM + Zn	125.59 ± 1.8a	123.46 ± 2.1a	124.73 ± 4.5a
Pr + CCM + Zn + PA	82.14 ± 1.2b	84.11 ± 0.2b	86.00 ± 2.3b

[a] Means and standard deviations for a minimum of three replicates.
[b] Pr = protein, Ca = $CaCO_3$, CCM = calcium-citrate-malate complex, Zn = zinc and PA = phytate.
[c] Means within columns and pH level with different lower case letters are significantly different at $p < 0.05$.

served in the Pr + PA treatment with soy concentrate and casein at pH 5.5 (Table 4). This same effect was observed by Grynspan and Cheryan (1989). Not only did protein solubility increase but phytate solubility increased, an effect noted by Prattley et al. (1982) indicating the dissolution of the insoluble protein-phytate complex with increased pH. Prattley et al. (1982) speculated that resulted from the repulsion of negatively charged phytate by the increasingly negative protein or from the conformational changes in the protein itself. A significant increase in soluble protein from the following casein systems was also observed, Pr + Ca ($P < 0.05$) and Pr + Ca + Zn, Pr + CCM + Zn and Pr + Zn + PA ($P < 0.01$).

However, at pH 5.5 no differences were observed among systems with soy concentrate or yeast indicating the presence of calcium, zinc, phytate or any combination of these did not affect protein solubility. However, with casein those treatments containing both calcium and phytate significantly decreased ($P < 0.01$) soluble protein due to formation of protein-calcium-phytate complexes as indicated by reduction in both soluble calcium and phytate. The exception to this was the Pr + CCM + Zn + PA treatment where the reduction in protein solubility was not significant.

Soluble calcium

The addition of phytate to the proteins did not alter endogenous calcium solubility at either pH level probably because of the low levels of calcium present. When calcium was added either as $CaCO_3$ or CCM no differences were observed in calcium solubility at pH 2.0 and the presence of zinc and/or phytate did not alter this (Table 5).

Increasing the pH from 2.0 to 5.5 significantly decreased ($P < 0.01$) calcium solubility in all the phytate containing treatments presumably by the co-precipitation of calcium and phytate in an insoluble complex (Table 5). Increasing the pH also significantly reduced ($P < 0.01$) calcium solubility in the Pr + Ca + Zn treatment with soy concentrate, and the Pr + Ca treatment with yeast.

Calcium solubility was significantly lower ($P < 0.01$) in treatments containing phytate at pH 5.5 with all protein sources. An insoluble phytate-calcium complex presumably formed as evidenced by significant reductions ($P < 0.01$) in solubility for both phytate and calcium. Whether this insoluble complex also combined with protein is debatable. Kroll (1984) found as pH increased above pH 3 ionizable groups of the protein became deprotonated and bound calcium. This was in contrast to other studies which reported no binding of calcium to protein at pH 5.5 (Appu Rao and Narasinga Rao 1975) or at pH 7.0 (Sakakibara and Noguchi, 1977). The ability of protein to bind calcium may depend on the degree of dissociation, the conformational changes that accompany the dissociation (Kroll, 1984) and the source of the protein (Champagne, 1988). Grynspan and Cheryan (1989) found calcium interacted with insoluble protein which decreased calcium solubility without altering protein solubility. Further studies are required to determine whether the phytate-calcium complex can bind to protein.

Table 6—Means and standard deviations of soluble zinc from three different protein sources; casein, soy concentrate and torula yeast, with added zinc and with and without added calcium and phytate at pH 2.0 and 5.5

Treatments[b]	Soluble zinc (μg/g protein)[a] Protein sources[c] Casein	Soy concentrate	Yeast
pH 2.0			
Pr	36.80 ± 1.3b	54.65 ± 0.0c	373.99 ± 3.7de
Pr + PA	56.26 ± 6.2b	75.65 ± 9.6c	350.41 ± 6.4e
Pr + Ca	48.70 ± 0.0b	not determined	382.59 ± 5.5cde
Pr + Ca + PA	not determined	not determined	416.86 ± 3.7cd
Pr + Zn	325.78 ± 9.9a	350.41 ± 5.2a	654.75 ± 9.8ab
Pr + Zn + PA	309.70 ± 3.7a	310.23 ± 15.2b	618.32 ± 3.7b
Pr + Ca + Zn	315.77 ± 15.3a	340.77 ± 11.1a	641.89 ± 7.4ab
Pr + Ca + Zn + PA	323.09 ± 13.2a	337.56 ± 10.5a	646.18 ± 6.4ab
Pr + CCM	not determined	not determined	446.86 ± 0.0cd
Pr + CCM + PA	not determined	not determined	385.78 ± 4.6de
Pr + CCM + Zn	340.78 ± 4.6a	334.34 ± 4.6a	684.76 ± 9.1a
Pr + CCM + Zn + PA	331.13 ± 9.1a	321.48 ± 4.6a	662.26 ± 4.6ab
Total Ca + Zn	322.49 ± 9.1a	357.68 ± 8.4a	not determined
Total CCM + Zn	343.98 ± 9.1a	343.98 ± 9.1a	not determined
pH 5.5			
Pr	not determined	24.35 ± 3.2b	139.62 ± 11.9ef
Pr + PA	38.15 ± 4.9c	50.87 ± 3.8b	113.64 ± 0.0efg
Pr + Ca	12.98 ± 4.6c	not determined	71.44 ± 13.5efg
Pr + Ca + PA	not determined	not determined	37.88 ± 3.7fg
Pr + Zn	123.38 ± 15.5b	81.17 ± 9.2b	245.14 ± 21.5c
Pr + Zn + PA	156.94 ± 9.9ab	133.13 ± 14.2a	233.78 ± 16.3cd
Pr + Ca + Zn	36.53 ± 8.1c	30.84 ± 6.2b	161.27 ± 3.7de
Pr + Ca + Zn + PA	17.86 ± 8.2c	32.47 ± 6.5b	22.67 ± 5.3g
Pr + CCM	not determined	not determined	321.35 ± 4.6b
Pr + CCM + PA	not determined	not determined	45.91 ± 4.6eg
Pr + CCM + Zn	186.91 ± 0.0a	59.02 ± 4.6b	446.56 ± 36.1a
Pr + CaCM + Zn + PA	22.95 ± 0.0c	26.23 ± 4.6b	45.92 ± 4.6efg

[a] Means and standard deviations for a minimum of three replicates.
[b] Pr = protein, Ca = $CaCO_3$, CCM = calcium-citrate-malate complex, Zn = zinc, PA = phytate.
[c] Means within columns and pH levels with different lower case letters are significantly different at $p < 0.05$.

Table 7—Means and standard deviations of soluble phytate from three different protein sources; casein, soy concentrate and torula yeast, with added phytate and with and without added calcium and zinc at pH 2.0 and 5.5.

Treatments[2]	Soluble phytate (mg/g protein)[1] Protein sources[3] Casein	Soy concentrate	Yeast
pH 2.0	±	±	±
Pr + PA	30.37 ± 3.6c	37.68 ± 1.0a	53.42 ± 3.5a
Pr + Ca + PA	65.80 ± 5.8a	51.88 ± 9.6a	60.74 ± 4.5a
Pr + Zn + PA	33.74 ± 0.0bc	42.13 ± 9.1a	55.67 ± 1.7a
Pr + Ca + Zn + PA	64.11 ± 15.2a	55.68 ± 13.7a	71.98 ± 3.9a
Pr + CCM + PA	60.73 ± 5.8ab	68.05 ± 6.8a	82.67 ± 2.4a
Pr + CCM + Zn + PA	70.86 ± 0.0a	50.62 ± 2.4a	82.10 ± 10.8a
pH5.5			
Pr + PA	99.54 ± 14.0a	127.66 ± 8.7a	96.17 ± 10.3a
Pr + Ca + PA	0.00 ± 0.0b	0.00 ± 0.0b	0.00 ± 0.0b
Pr + Zn + PA	74.24 ± 2.4a	146.07 ± 24.9a	83.93 ± 20.8a
Pr + Ca + Zn + PA	0.00 ± 0.0b	0.00 ± 0.0b	0.00 ± 0.0b
Pr + CCM + PA	0.00 ± 0.0b	0.00 ± 0.0b	0.00 ± 0.0b
Pr + CCM + Zn + PA	0.00 ± 0.0b	0.00 ± 0.0b	0.00 ± 0.0b

[1] Means and standard deviations for a minimum of three replicates.
[2] Pr = protein, Ca = $CaCO_3$, CCM = calcium-citrate-malate complex Zn = zinc and PA = phytate.
[3] Means within columns and pH level that have different letters are significantly different at $p < 0.05$.

Soluble zinc

The addition of phytate to soy protein concentrate slightly increased endogenous soluble zinc at both pH 2.0 and pH 5.5 but did not alter its solubility with yeast or casein (Table 6). The presence of phytate in the Pr + Zn + PA treatment of soy concentrate resulted in a highly significant reduction ($P < 0.01$) in solubility of both protein (Table 4) and zinc (Table 6). However, its addition did not alter zinc solubility in other protein sources at pH 2.0. The presence of calcium did not alter zinc solubility at low pH with any of the proteins (Table 6). Increasing the pH resulted in a highly significant decrease ($P < 0.01$) in zinc solubility in all treatments with all proteins (Table 6).

At pH 5.5 significant differences in zinc solubility were also observed due to treatment effects. Zinc solubility from soy concentrate was significantly greater ($P < 0.05$) with phytate than without it. This may have been due to conformational changes in the protein due to its interactions with phytate.

$CaCO_3$ significantly reduced ($P < 0.01$) zinc solubility in all proteins, both with and without phytate. CCM in the absence of phytate significantly enhanced ($P < 0.01$) zinc solubility with casein and yeast but did not affect zinc solubility with soy concentrate. The CCM complex dissociates at low pH and zinc may have formed a soluble complex with citrate and/or malate when the pH increased with prevented it from being bound in an insoluble complex. Citrate appeared to have a stronger affinity for zinc than for calcium, probably as a result of the ability of transition metals to form coordinate complexes with ligands (Kratzer and Vohra, 1986). Citrate has been found to compete with casein for zinc forming a soluble citrate-zinc complex (Harzer and Kauer, 1982). The processing conditions of soy concentrate used were unknown and could have contributed to the binding of zinc to the protein. The addition of phytate to the CCM treatments resulted in a significant decrease ($P < 0.01$) in zinc solubility with all proteins.

Soluble phytate

No endogenous phytate was detected in any protein source. Therefore, these experiments investigated the effects of added sodium phytate on the solubility of zinc, calcium and protein. The effects observed here would not necessarily be the same for endogenous phytate. Significantly less ($P < 0.01$) soluble phytate was observed with casein when the Pr + PA treatment was compared to all the treatments containing calcium at pH 2.0 (Table 7). This agrees with the observations of Champagne (1988) and Okubo et al. (1976) who noted that cations could disrupt the protein-phytate interaction at low pH. With casein, phytate solubility in the treatment containing zinc was significantly lower ($P < 0.01$) than those containing calcium, calcium and zinc or CCM and zinc but not different than that with CCM. However, no significant differences in soluble phytate were observed with the other protein sources when calcium and/or zinc were present. Increasing pH to 5.5 significantly increased ($P < 0.01$) phytate solubility in the Pr + PA treatment with all proteins and the Pr + Zn + PA treatment for soy concentrate (Table 7). The pH increase probably caused partial dissociation of protein-phytate complex which resulted in increased solubility for both protein and phytate.

No soluble phytate was detected in the treatments that contained calcium at pH 5.5. Insoluble complexes were formed when phytate was present in all treatments at pH 5.5. With the casein protein, solubility of all components was significantly reduced ($P < 0.01$) indicating formation of an insoluble protein-phytate-mineral complex. With soy concentrate and yeast, protein solubility was unaltered. However, the solubility of calcium, zinc and phytate was significantly reduced indicating these components apparently precipitated in an insoluble complex. Whether this insoluble complex bound to previously precipitated protein is unknown.

CONCLUSIONS

THE pH was an extremely important factor in determining the final solubility of the components. At pH 2.0 added calcium and zinc were completely soluble and not affected by protein source or phytate. An increase in pH significantly reduced ($P < 0.01$) zinc solubility in all treatments. Calcium solubility was also significantly reduced ($P < 0.01$) but only when phytate was present. Phytate solubility decreased significantly ($P < 0.01$) at pH 5.5 when calcium was present. Increasing the pH also decreased protein solubility significantly ($P < 0.01$) whether with

protein alone, or protein and zinc, for both soy concentrate and casein. A nonsignificant reduction was seen for yeast. In the phytate treatment, protein solubility was increased as the pH was raised with casein and soy concentrate.

The ability of food proteins to bind phytate or minerals appears to be related to its source. In a recent review Champagne (1988) stated that some proteins bind phytate while others do not. O'Dell and de Boland (1976) suggested the amino acid composition was not the key component in binding, but the accessibility of the binding groups that determined phytate binding to protein. Also an increase in dietary protein decreased phytate-zinc binding (Mills, 1985).

Addition of zinc did not affect solubility of other components, perhaps, because of its low concentration. When phytate and/or calcium were in the treatment their effects dominated. This agreed with the suggestion by Mills (1985) that the calcium determined zinc availability in phytate rich diets and had a synergistic effect on phytate-zinc antagonism.

Yeast contained considerably more endogenous zinc than the other two proteins. No differences were observed in effects of added calcium and phytate on endogenous or exogenous zinc.

The results found here indicate the importance of chemical interactions in mineral solubility and potential bioavailability. Such interactions must be understood and documented if appropriate decisions are to be made in the fortification of formulated food in order to maximize nutritional effects.

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Effect of Heat and pH on Toxigenic *Clostridium butyricum*

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ABSTRACT

Clostridium butyricum long known to cause spoilage in canned acid foods recently caused two separate cases of infant botulism in Italy by strains producing type E botulinum toxin. Heat resistance of spores of toxigenic and nontoxigenic *C. butyricum* was determined. The nontoxigenic strain was considerably more heat resistant, having a D-value at 212°F of 4.7 min compared to a D-value 170°F of 2.3-2.5 min for toxigenic strains at pH 7.0. Minimum pH for growth and toxin production was also determined. The nontoxigenic strain grew at pH 4.2; toxigenic strains grew and produced toxin at pH 5.2 but not at pH 5.0.

INTRODUCTION

CLOSTRIDIUM BUTYRICUM is one of a few sporeforming anaerobes capable of growth in acid foods. It has been responsible for spoilage in acid canned foods (pH≤4.6), especially tomatoes (Townsend, 1939). If a large number of these organisms are present, some could survive heat processes used on canned acid foods and cause spoilage.

Until recently, *C. butyricum* was considered a nonpathogenic species. In 1986, two separate cases of infant botulism occurred in Italty (Aureli et al., 1986) caused by organisms producing toxin neutralized by type E *C. botulinum* anti-toxin, although the organisms were phenotypically similar to *C. butyricum* (McCroskey et al., 1986). DNA homology studies also indicated the toxigenic strains were *C. butyricum*. The binding ratio of the DNA of a toxigenic strain with DNA of a *C. butyricum* strain was 75% but only 6–12% with DNA of *C. botulinum* strains (Suen et al., 1988). Gimenez and Sugiyama (1988) have shown the primary molecular properties of the *C. butyricum* toxin are very similar but not identical to type E botulinum toxin.

The objective of our study was to determine heat resistance of spores and minimum pH for growth and toxin formation by toxigenic *C. butyricum*.

MATERIALS & METHODS

Organisms

Toxigenic *C. butyricum* strains 5262 and 5520 were isolated from infant botulism cases and were obtained from the Centers for Disease Control (CDC). A nontoxigenic strain (87-409) was isolated in our laboratory from spoiled blueberries. The organisms were confirmed biochemically as *C. butyricum* by the methods described by Holdeman et al. (1977).

Production of spores

Spores of strain 5262 and 5520 were grown anaerobically in GasPak jars (BBL) on modified dextrose tryptone agar (Difco) containing 0.1% potassium phosphate and 0.1% manganese sulfate (DTA). DTA yielded consistently high counts of spores. DTA was a good growth media for 87-409 but proved not to be a good medium for sporulation; therefore, spores of strain 87-409 were produced on Keener's Medium (honey, 4g; yeast extract, 4g; malt extract, 4g; agar, 14 g per 1 liter H_2O) in GasPak jars. Spores were harvested (after 2 wk incubation at 30°C) by centrifugation, washed twice and stored at 4°C in sterile distilled water. Spore counts were determined by heat shocking at 60°C for 10 min, plating on DTA and incubating in GasPak jars.

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Determination of heat resistance

Heating menstrua. Spores of the three strains were tested at different pH levels. Death rates at neutral pH were determined with spores in Sorenson's M/15 phosphate buffer (0.067M, pH 7.0). Heat resistance in acid was determined with dextrose tryptone broth (DTB; NFPA, 1968a) acidified in 0.3 pH unit increments from pH 5.0 to pH 3.5 with 10% citric acid and commercially sterile tomato juice, purchased at a local supermarket, and adjusted to pH 4.8, 4.4 and 4.0 with 10% citric acid or 1N sodium hydroxide.

Thermal death time testing. Thermal death time (TDT) testing was carried out by a modification of TDT procedures outlined by NFPA (1968b). Modifications included use of ten TDT tubes per time interval rather than four and 1 mL per TDT tube rather than 2 mL. Spores were diluted in 400 mL heating menstruum to about 1×10^4 spores/mL. One mL of suspension was transferred to each pyrex glass TDT tube (8mm i.d. ×10cm) to be heated. The tubes were heat sealed, then heated while submerged in an oil bath.

The heated suspension was transferred to deaerated PE-2 medium (Folinazzo and Troy, 1954), overlaid with sterile vaspar (9 parts vaseline, 1 part mineral oil) and incubated at 30°C. The PE-2 cultures were incubated 6 months. Smears of cultures from the longest time interval exhibiting growth at each temperature were examined to confirm presence of the test organism. In addition, for toxigenic strains culture supernatants from the longest time interval showing growth, and first time interval with no growth, at each temperature were tested for toxicity by the mouse assay (FDA, 1984) at the end of 6 months.

Heating times and temperatures appropriate for each menstruum-pH combination were determined by preliminary trials. One TDT test consisted of 300 tubes: ten tubes at each of six different time intervals at each of five temperatures. Temperatures for strains 5262 and 5520 ranged from 165°F to 182.5°F and for strain 87-409 from 204°F to 224°F. Temperature data were measured with a copper constantan thermocouple located in the approximate center of the inoculum in a TDT tube. Data from the thermocouple were recorded at 10-sec intervals using a Kaye Digistrip II potentiometer and a DEC Mini-MINC computer.

Data were analyzed using the TDT programs developed by NFPA. The computer program used the general method (Stumbo, 1973) to establish cumulative lethality for each heating time interval. This corrected time of lethality was used in Stumbo's formula (1973) to calculate the decimal reduction (D) value at each temperature:

$$D = \frac{t}{\log A - \log B}$$

where t = corrected lethality in minutes; A = initial spore concentration; B = number of surviving spores (assuming one viable organism per tube).

The D-values calculated for the heating temperatures were plotted to obtain a phantom TDT curve. The z-value was obtained from the slope of the curve.

Growth study

After pH adjustment, DTB (10 mL) and tomato juice (10 mL) were inoculated with 10^4 heat shocked spores (60°C, 10 min). Ten separate tubes of pH adjusted DTB and tomato juice were inoculated with each *C. butyricum* strain; these were overlaid with sterile vaspar and in-

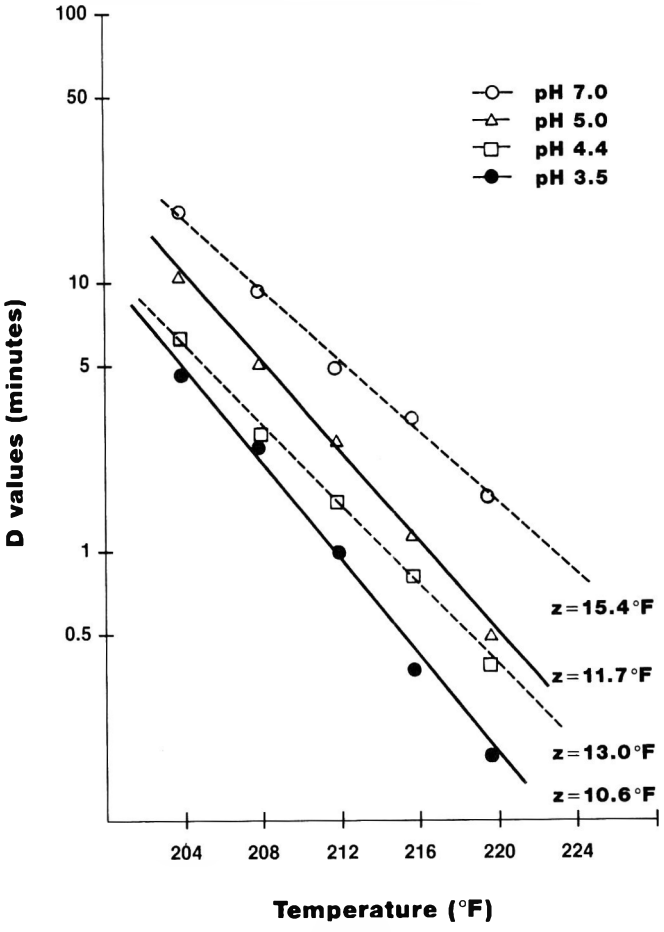

Fig. 1.—A comparison of thermal death time curves for C. butyricum *strain 87-409 heated in phosphate buffer (pH 7.0) and dextrose tryptone broth at pH levels of 5.0, 4.4 and 3.5.*

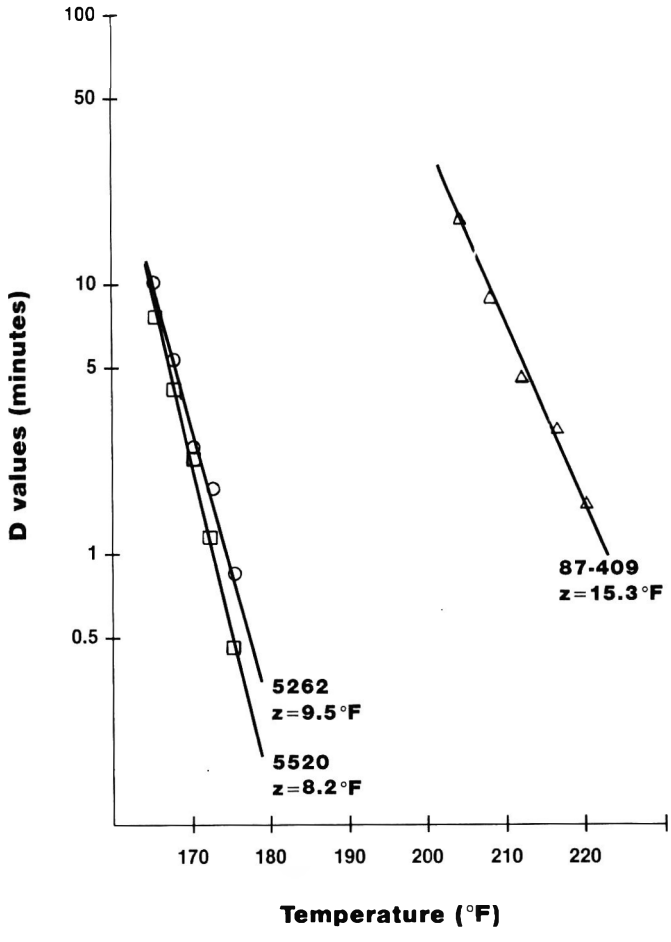

Fig. 2.—A comparison of thermal death time curves for C. butyricum *strains 5262, 5520 and 87-409 heated in phosphate buffer (pH 7.0).*

Table 1—Growth of C. butyricum *strains 5262, 5520 (toxigenic) and 87-409 (nontoxigenic) in dextrose tryptone broth acidified to different pH levels*

	Results (# positive tubes/total)		
pH	87-409[a]	5262[b]	5520[c]
5.4	10/10	10/10	10/10[d]
5.2	10/10	10/10[d]	1/10[d,e]
5.0	10/10	0/10[f]	0/10[f]
4.8	10/10	0/10	0/10
4.6	10/10	0/10	0/10
4.4	10/10	0/10	0/10
4.2	0/10	0/10	0/10

[a] Inoculum = 2.1 × 10^4 spores/10 mL
[b] Inoculum = 4.4 × 10^4 spores/10 mL
[c] Inoculum = 1.2 × 10^4 spores/10 mL
[d] Confirmed positive for toxin by the mouse bioassay
[e] Growth without gas
[f] Confirmed negative for toxin by the mouse bioassay

Table 2—D-values (min) of C. butyricum *strain 87-409 heated in pH-adjusted tomato juice and phosphate buffer (pH 7.0)*

	Temperature (°F)						z
pH	204	208	212	216	220	224	(°F)
7.0[a]	17.3	9.1	4.7	3.0	1.50	ND[c]	15.4
4.8[b]	ND[c]	4.9	2.8	1.4	0.90	0.42	15.3
4.4[b]	ND[c]	3.3	1.8	1.1	0.54	0.35	16.3
4.0[b]	ND[c]	2.5	1.3	0.74	0.38	0.15	13.6

[a] Phosphate buffer
[b] Tomato juice
[c] Not determined

cubated at 30°C. The presence of gas and/or turbidity indicated a positive culture. Positive cultures inoculated with strains 5262 and 5520 were trypsinized to activate toxin (FDA, 1984) and tested for toxicity by the mouse assay. Toxin testing was also performed on cultures showing no growth to confirm negatives. All cultures were incubated 9 months, which was at least 60 days after the last positive observation.

RESULTS & DISCUSSION

Growth Inhibiting pH study

Strains 5262 and 5520 grew in DTB acidified to pH 5.2 but not to pH 5.0 (Table 1). Strain 5262 exhibited turbidity, gas production and toxin production in all ten tubes at pH 5.2. The one tube at pH 5.2 in which strain 5520 grew became turbid without gas production but tested positive for toxin. Toxin was not present in DTB that did not become turbid. Strains 5262 and 5520 did not grow in tomato juice at the highest pH (4.8) used in this study (data not shown). *C. butyricum* strain 87-409 grew in DTB of pH 4.4 but not 4.2 (Table 1). Strain 87-409 grew in tomato juice at pH 4.2 but not at 4.0.

Butyric anaerobes are considered somewhat acid tolerant and capable of growth in foods with pH levels in the range of 4.0 or greater (Townsend, 1939). These studies indicate the toxigenic strains of *C. butyricum* tested cannot grow at pH 5.0 or below.

Heat resistance

Strain 87-409 had a $D_{212°F}$ in phosphate buffer (pH 7.0) of 4.7 min and a $D_{212°F}$ in tomato juice (pH 4.0) of 1.3 min (Table 2). The heat resistance of this strain was somewhat lower in acidified DTB than that observed in tomato juice (data not shown). In general, heat resistance of this strain decreased as the pH decreased, as shown in Fig. 1. Townsend (1939) also

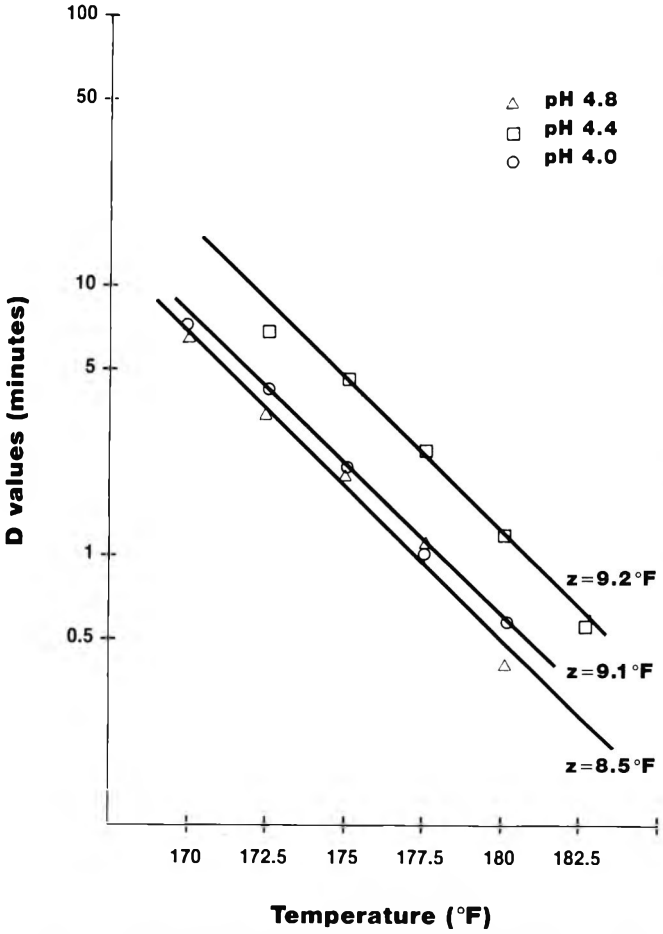

Fig. 3.—Thermal death time curves for C. butyricum *strain 5262 heated in tomato juice at pH levels of 4.8, 4.4 and 4.0.*

Table 3—D-values (min) of C. butyricum *strain 5262 heated in dextrose tryptone broth acidified to different pH levels*

	Temperature (°F)					z
pH	167.5	170	172.5	175	177.5	(°F)
5.0	10.9	6.0	2.9	1.7	0.68	8.5
4.7	12.6	7.0	3.6	1.8	0.90	8.7
4.4	17.5	8.6	4.0	1.7	1.0	7.8
4.1	11.1	7.1	3.2	1.6	0.66	8.0
3.8	9.6	4.9	2.1	1.0	0.45	7.5
3.5	7.2	3.7	1.7	0.84	0.57	8.8

Table 4—D-values (min) of C. butyricum strain 5520 heated in dextrose tryptone broth acidified to different pH levels

	Temperature (°F)						
pH	165	167.5	170	172.5	175	177.5	z
5.0	13.6	6.0	2.7	1.4	0.81	a	8.1
4.7	—[a]	11.8	5.7	3.0	1.0	0.94	8.5
4.4	—[a]	13.5	7.1	3.4	1.2	0.90	8.0
4.1	—[a]	11.7	7.1	3.8	1.9	1.0	9.3
3.8	—[a]	9.7	4.5	2.1	1.8	0.59	8.9
3.5	—[a]	6.8	3.9	1.5	0.74	0.33	7.4

[a] Not determined

observed that as the pH decreased, the heat resistance of spores of butyric anaerobes decreased. He reported a $D_{212°F}$ of 6 min in phosphate buffer; in tomato juice the $D_{212°F}$ was 2.1 min at pH 4.5 and 1.2 min at pH 3.8.

The spores of strains 5262 and 5520 were much lower in heat tolerance than that of the nontoxigenic strain 87-409. The TDT curves for all three strains when heated in phosphate buffer (pH 7.0) are compared in Fig. 2. Strain 5262 had a $D_{170°F}$ of 2.5 min and strain 5520 a $D_{170°F}$ of 2.3 min. Extrapolation of D-values for strain 87-409 indicates that at 170°F the nontoxigenic strain would have a D-value over 1000 times greater than the toxigenic strains. D and z-values for the toxigenic strains in acidified DTB are listed in Tables 3 and 4. The highest heat resistance for both strains occurred at pH 4.1-4.4 in DTB. This was also true in general in tomato juice (Fig. 3 and 4) where the heat tolerance was greatest at pH 4.4. While the heat resistance of these toxigenic strains was lower than 87-409, the optimal heat resistance did not appear to follow the same pattern as that of nontoxigenic strains.

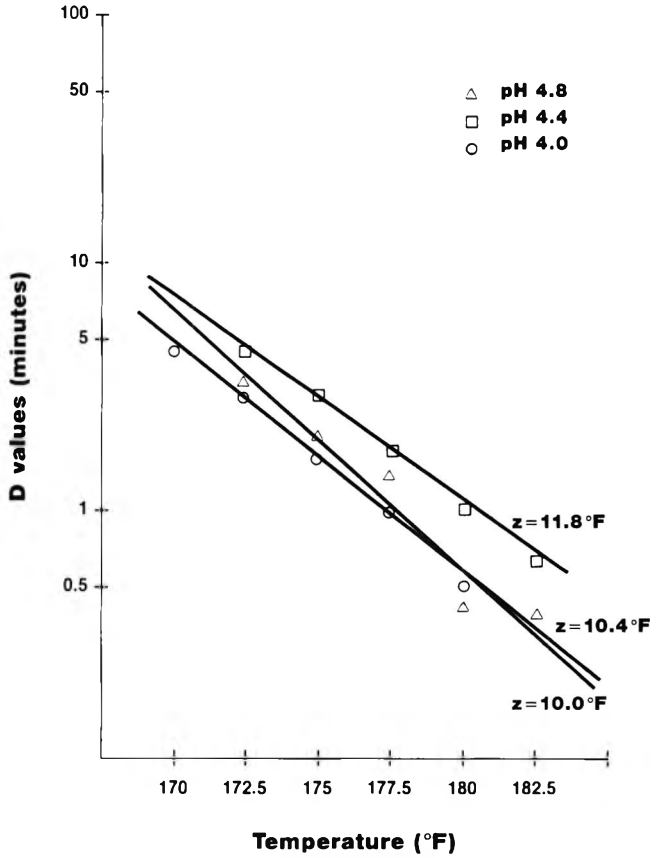

Fig. 4.—Thermal death time curves of C. butyricum *strain 5520 heated in tomato juice at pH levels of 4.8, 4.4 and 4.0.*

CONCLUSIONS

OUR STUDY indicated toxigenic strains of *C. butyricum* were considerably less heat resistant than the nontoxigenic strain we tested, as well as other reported strains.

In addition, the toxigenic strains could not grow at pH levels ≤ 5.0. Given these results, the toxigenic strains apparently would not survive the usual heat processes for acid foods or grow at pH levels normally associated with acid foods. Therefore, the toxigenic *C. butyricum* strains 5262 and 5520 should not be a concern in such products.

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Tetrathionate Protects Proteolytic Activity of Simulated Papaya Latex and Crude Papain

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ABSTRACT

Sodium tetrathionate (TT) was assessed for its possible protection of proteolytic activity (PA) of rehydrated crude papain during drying and storage. The addition of 1% TT significantly decreased the loss of PA of rehydrated crude papain after being oven dried at 55°C. This protection of PA was 15–20% higher than that from metabisulfite. Crude papain with 1% TT lost 20% of PA and with metabisulfite lost 45% of PA in 13 wk at room temperature. Results suggest TT may decrease loss of PA during papaya latex processing and storage of crude papain.

INTRODUCTION

ENZYMES, unlike many other substances, are standardized and sold by their activity rather than their weight. The stability of an enzyme during its isolation, purification, formulation and storage is therefore of prime importance (Cheetham, 1985). During manufacturing of commercial papain a significant loss of proteolytic activity (PA) occurs. This loss mainly occurs during harvesting and drying of fresh papaya latex and during subsequent storage of dry papaya latex (Brocklehurst et al., 1981; Jones and Mercier, 1974; Poulter and Caygill, 1985) commonly referred to as crude papain (Poulter and Caygill, 1985).

The PA of crude papain (i.e. dry papaya latex) and of commercial papain (i.e. partially purified crude papain) determines its commercial value in a wide range of applications (Narinesingh and Mohammed-Naraj, 1988; Miles, 1985). Thus, losses of PA in manufacturing commercial papain bring about direct economic losses to the producer. The major causes of PA losses in papain preparations are: irreversible oxidation of essential cysteine residues in the proteases and autolysis of these enzymes (Ortiz et al., 1980; Brocklehurst et al., 1981).

The PA losses due to oven drying have been reported to be 7 ± 2% (Ortiz et al., 1980) to 20% (CONAFRUT, 1973). Sun-dried crude papain had 20–32% less milk clotting activity compared with vacuum-dried crude papain (Krishnamurty et al., 1960). Castro (1981) reported the loss of PA due to storage of crude papain over a period of 75 days was 30% at room temperature and 25% refrigerated.

Addition of reducing agents, such as sodium bisulfite or metabisulfite, in combination with a chelating compound (i.e. EDTA) before drying, decreased losses of PA due to drying as compared to an untreated sample (Ortiz et al., 1980; Krishnamurty et al., 1960).

Sodium tetrathionate, a symmetrical disulfide, reversibly oxidized thiols quantitatively to their corresponding disulfides. The oxidation was promptly reversed by addition of a reducing agent such as cysteine, β-mercaptoethanol or dithiothreitol (Means and Feeney, 1971; Pihl and Lange, 1962). Upon reacting with tetrathionate, the sulfhydryl protease became reversibly inactive. Thus, the process of autolysis was inhibited. Tetrathionate also protects the active cysteine residue in cysteine proteases from irreversible oxidation (Englund et al., 1968). Autolysis and oxidation are major causes of irreversible losses in enzymatic activity of cysteine proteases. Therefore we could expect sodium tetrathionate would increase stability of papaya latex by decreasing PA losses during drying and storage.

Tetrathionate has been used successfully to prevent autolysis during chromatographic purification of cysteine proteases (Takahashi et. al., 1973; Englund et al., 1968; Pal et al., 1984; Kitamura and Maruyama, 1986; Aworh and Nakai, 1988). Sodium tetrathionate also has an antimicrobial effect against some bacteria (Palumbo and Alford, 1970). It was possible that addition of tetrathionate to papaya latex might decrease the microbial load of latex.

The objective of our study was to evaluate a new method to stabilize PA during drying of rehydrated crude papain and during its subsequent storage. We also tested the addition of sodium tetrathionate for control blocking or oxidation of sulfhydryl groups of the proteases in rehydrated crude papain. Finally we wanted to compare protection of the PA provided by tetrathionate to that produced by sodium metabisulfite, commonly used in commercial drying of papaya latex.

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MATERIALS & METHODS

Materials

Fresh papaya latex is commercially unavailable. Crude papain, Type I P3250 (Sigma Chemical Co., St. Louis, MO) was used. According to the chemical supplier this crude papain is "dried papaya latex. . . not cleaned or purified." Sodium tetrathionate was obtained from ICN Pharmaceuticals, Inc. (Life Science Group, Plainview, NY) and sodium metabisulfite, anhydrous powder, was purchased from Matheson Coleman & Bell Manufacturing Chemists, Norwood, OH.

Rehydration of crude papain

To simulate fresh papaya latex, Sigma crude papain Type I was rehydrated with distilled water to a 20% solution (w/v), a solid content similar to fresh papaya latex (Narinesingh and Mohammed-Naraj, 1988; Ortiz et al., 1980). The rehydration was as follows: crude papain was mixed with cold distilled water and left at 4°C 2 hr with slow agitation.

Drying characteristics of rehydrated crude papain

Variables studied were: temperature (55, 80 and 100°C), drying load (1,1990; 2,381; and 4,792 g/m^2 of drying area), and addition of sulfite or tetrathionate (both at 1% w/v). Parallel experiments with control samples (i.e. samples with no additives) were also performed. An electrically heated, mechanical convection, forced horizontal air-flow tray drier was used (Blue M Stabil-Therm Oven, Model OV-490a-2, Blue M Electric Company, Blue Island, IL).

Samples were dried in aluminum dishes (i.d. 2.3 cm and 0.5 cm deep). The oven was preheated for ≥ 1 hr at the defined temperature before starting drying experiments. To obtain drying curves, samples were withdrawn from the oven at various time intervals, and moisture content of all samples was determined as weight loss after 12 hr at 80°C under vacuum.

Determining influential factors on losses of PA

The L_{27} (3^{13}) fractional factorial design of Taguchi (1987) was used to determine factors which may significantly affect losses of PA due to drying. In addition, using this experimental design we determined if tetrathionate protected the PA of rehydrated crude papain during drying, the degree of protection, and compared it with that from so-

Table 1—Factors and assigned levels investigated for possible influence on losses of proteolytic activity of rehydrated crude papain

Factor	Level 1	2	3
Predrying treatment[a]	TT	MBS	CON
Drying temperature, °C	55	70	100
Storage prior to drying, hr	2	12	24
Drying load, g/m²	1,190	2,381	4,762
Type of storage prior to drying[a]	D	D/L	L

[a] Predrying treatments: TT = 1% sodium tetrathionate; MBS = 1% sodium metabisulfite; CON = no additives.
[a] Type storage prior to drying: D = storage in the dark; D/L = first half storage dark and other half light; L = storage under light.

dium metabisulfite. The L_{27} table was composed of 27 rows of an orthogonal combination of three level numbers for 13 columns, to assign factors and their interactions for up to 12 columns. From the letter assigned for each column (called "component") we could readily determine which columns had between-factor interactions. Factors evaluated, together with the three levels assigned to each, are shown in Table 1.

The pre-drying additive, sodium metabisulfite or sodium tetrathionate, was added directly to the rehydrated crude papain. The sample was stored at room temperature (20–22°C) prior to drying under the conditions specified by the fractional factorial design. After storage samples were dried in the forced air oven, at the temperatures specified. Drying was continued until the latex hardened and crumbled readily when pressed by fingers. This usually occurred when residual moisture was 6±2%.

Dried samples were sealed in polyethylene bags. The bags were placed inside amber glass jars with desiccant (Drierite) and stored at -20°C until PA determination (within 3 wks). Preliminary experiments showed no losses of PA occurred during this storage.

Data collected from the 27 drying treatments (in duplicate), following the fractional experimental design L_{27} (3^{13}) of Taguchi (1987), were analyzed by analysis of variance to determine significance of the factors and possible interactions on PA losses during drying using a Taguchi factorial analysis of variance computer program written in IBM-BASIC (Arteaga, 1986).

Effect of drying type and additives on losses of PA

The effect of three drying types, namely, sun, vacuum and oven drying, on the losses of PA with or without additives was determined using a 3 x 3 full factorial experimental design. The two factors and corresponding levels, in parenthesis, were: type of drying (sun, vacuum, and oven drying) and treatment prior to drying (1% MBS, 1% TT, and no additives CON).

Additives were added directly to rehydrated crude papain. All samples were dried in aluminum dishes with a drying load of 1,190 g/m². Each treatment was in triplicate.

Sun drying (20–25°C) was carried out 6 hr. After that time, moisture content was still high (30%), so the samples were vacuum dried 3 hr at 55°C, 0.8 atm to moisture content 6±2%. Oven drying was done at 55°C for 1.5 hr, to reach final moisture 6±2%. Vacuum drying was 55°C at 0.8 atm 5 hr; final moisture was also 6±2%. Dried samples were sealed in polyethylene bags, and the bag placed inside amber glass jars with desiccant. The jars were stored at -20°C until PA determination (within 3 wk).

Losses of PA during storage

Rehydrated crude papain with 1% MBS or 1% TT was oven dried at 55°C 1.5 hr (drying load 1,190 g/m²). The resulting crude papain was sealed in polyethylene bags, and the bags placed in transparent glass jars and stored at room temperature.

At 1 wk intervals, up to 13 wk, PA was measured in triplicate. A control sample (i.e. crude papain with no additives was stored and analyzed in the same manner.

Proteolytic activity assay

The method of Hanada et al. (1987) with modifications was used. This is based on quantitation by uv spectrophotometry of trichloroacetic acid (TCA)-soluble peptides following casein hydrolysis. The casein solution (1% w/v) was prepared according to the Chemical Codex (FCC III, 1981), with the modification that Hammarsten-type casein was dissolved in 0.05M Tris-HCl buffer (pH 8.0) instead of phosphate-citrate buffer, pH 6.8. Use of Tris buffer enabled storage of the casein solution longer than 1 wk (Arnon, 1970). Proteolytic activities were expressed as international units i.e. μmols of tyrosine liberated $min^{-1}mg^{-1}$ of sample, under assay conditions (Hanada et al., 1978).

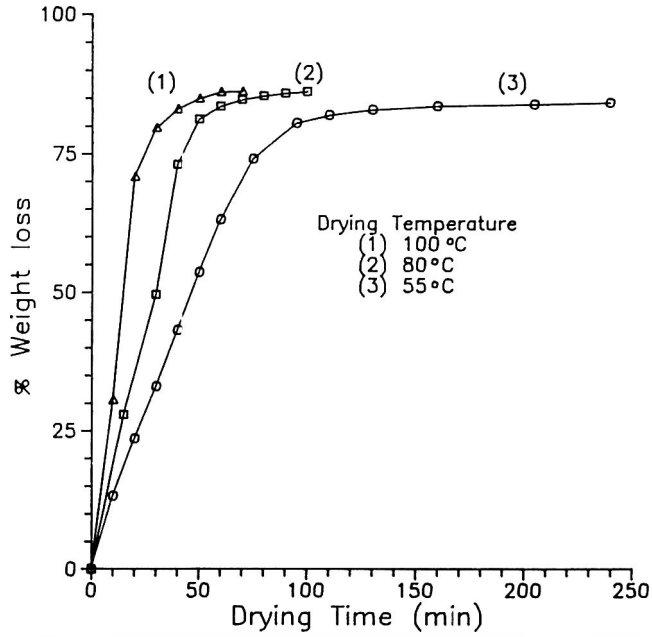

Fig. 1—Effect of drying temperatures on drying rate of rehydrated crude papain at drying load 2,381 g/m². Each point represents mean of three determinations. In all cases coefficient of variation was <5%.

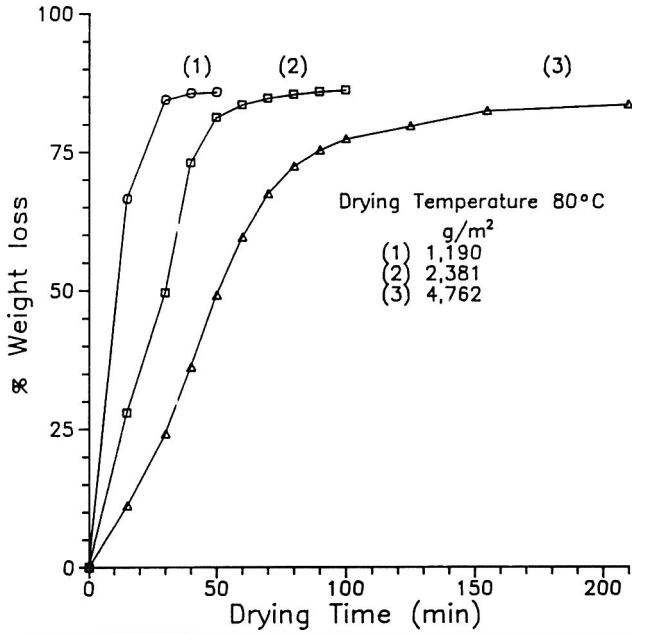

Fig. 2—Effect of load on drying rate of rehydrated crude papain at 80°C. Each point represents mean of three determinations. In all cases coefficient of variation was <5%.

RESULTS & DISCUSSION

Drying rates of rehydrated crude papain

Effects of temperature and drying load on drying rates of rehydrated crude papain, without additives, are shown in Fig. 1 and 2. As expected, the shortest time for reaching constant

Table 2—Analysis of variance (Taguchi's L_{27} 3^{13}) of proteolytic activity values of rehydrated crude papain latex from 27 experiments

Source of variation	DF	Mean square	F-value
Predrying treatment (PT)	2	730.91	13.64**
Drying temperature (DT)	2	9959.86	185.87**
Storage prior to drying	2	146.49	2.73 n.s.
Drying load	2	421.51	7.87**
Type of storage prior to drying	2	270.96	5.06*
PT × DT	4	311.17	5.81**
Error[a]	12	53.59	
Total	26		

[a] The sums of square values for interactions that do not appear in the ANOVA table were very low and, therefore, were incorporated into error sums of squares.
* significant at $p < 0.05$.
** significant at $p < 0.01$.
n.s. not significant at $p \geq 0.05$

Table 3—Mean retention of the proteolytic activity[a] (PA) of crude papain resulting from rehydrated crude papain treated with 1% tetrathionate of 1% metabisulfite and dried using different methods[b]

	Type of drying[c]		
Treatment	Sun	Vacuum	Oven
Control[d]	61.3 ± 2.6[e]	78.7 ± 3.5[f]	65.6 ± 3.5[e]
Metabisulfite	64.4 ± 3.2[e]	96.6 ± 3.0[g]	84.0 ± 3.9[h]
Tetrathionate	63.7 ± 3.1[e]	100.0 ± 3.8[g]	96.2 ± 4.0[g]

[a] % original PA present in the rehydrated crude papain.
[b] Each value = mean of three determinations. Mean ± S.D. Means not followed by same letter are significantly different at $p < 0.05$.
[c] See Materials and Methods
[d] no additives

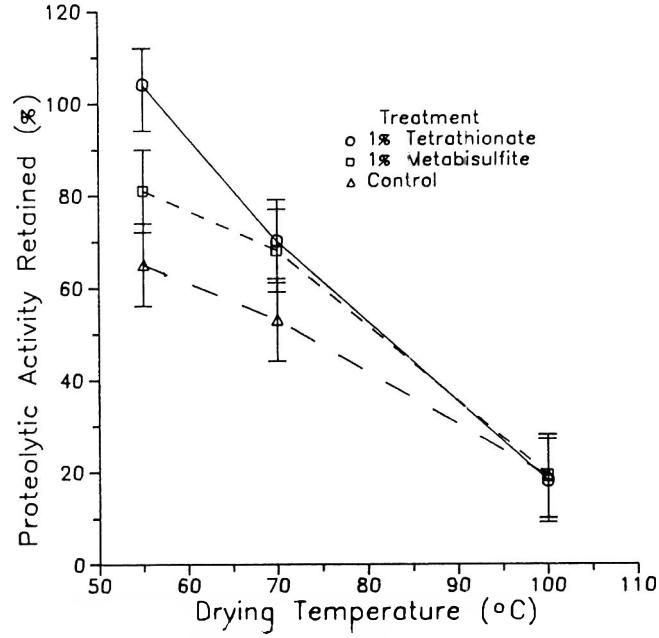

Fig. 3—Effect curve for interaction between drying temperature and treatment prior to drying on proteolytic activity retained by crude papain. (Mean ± Confidence limits calculated at $p < 0.05$).

weight was at the highest temperature (100°C), or the lowest load (1,190 g/m^2).

At 55°C, with drying load 2,381 g/m^2, about 150 min (2.5 hr) were required to reach constant weight (moisture content 6 ± 2%). Ortiz et al. (1980) reported fresh papaya latex required about 240 min to dry at 55°C. The difference in drying time is likely due to the different type oven. The shorter drying time for rehydrated crude papain, however, could indicate drying of papaya latex caused changes in some components of fresh papaya latex, with the result that added rehydratation water was not as tightly bound as the original water in the fresh papaya latex.

At all temperatures the addition of MBS or TT at 1% to rehydrated crude papain, did not change drying rate compared to a control.

Determining influential factors on loss of PA

Table 2 shows analysis of variance of the three level factorial design. The following main effects were computed to be highly significant ($p < 0.01$): treatment prior to drying, drying temperature and drying load. Storage conditions prior to drying (light or dark) had a significant effect at $p < 0.05$; storage in the dark prevented PA losses. The interaction (treatment prior to drying) x (drying temperature) was highly significant ($p < 0.01$). The other interactions were non significant ($p \geq 0.05$).

The effect curves in Fig. 3 illustrate the fact that mean PA retention was significantly higher with addition of 1% TT than with 1% MBS at 55°C. This was later confirmed by using a full factorial experimental design (next section). Although the mean value for PA after drying at 70°C was slightly higher when the crude papain was treated with TT than the corresponding value with MBS, overlapping of confidence limits ($p < 0.05$) indicated no significant difference between the two. Duncan's multiple range test confirmed this. At drying temperature of 100°C neither treatment protected PA.

Commercial drying of papaya latex is usually done at 50-55°C (Ortiz et al., 1980). Since TT was found significantly superior to MBS as a protecting agent of PA at 55°C, we concluded that TT has potential for commercial application as a protecting agent of papaya latex. For the control and for the rehydrated crude papain treated with either additive, highest PA mean retention occurred when drying load was minimum (1,190 g/m^2 drying area).

Effect of drying types and additives on loss of PA

As previously reported for fresh papaya latex (Krishnamurty et al., 1960), sun drying caused higher losses of PA in rehydrated crude papain as compared to other methods (Table 3). For the control sample the difference of PA between the resulting sun dried and oven dried crude papain was on the order of 20%, a value somewhat higher than the 10% reported by Castro (1981) for samples of crude papain from fresh papaya latex.

For sun drying addition of TT or MBS did not significantly decrease loss of PA compared to the control (i.e. no additives) sample. A major cause of PA loss during sun drying is thought to be the effect of UV radiation on a histidine residue essential for PA in the individual protease papain (E.C. 3.4.22.2) (Brocklehurst et al., 1981). Since neither TT nor MBS protect this histidine residue from UV radiation, a major decrease in losses of PA was not likely when TT or MBS was added.

As expected, vacuum drying produced minimum loss of PA in rehydrated crude papain (Table 3). Although oxidation was prevented with use of hypobaric conditions in this method, addition of TT or MBS before drying caused significant improvements in PA retention. Krishnamurty et al. (1960) reported addition of MBS at 0.5% before drying fresh papaya latex increased only slightly the retention of PA during vacuum drying as compared with an untreated sample. For oven drying at 55°C TT addition was significantly superior than to MBS addition (Table 3).

Effect of additives on loss of PA during storage

Addition of 1% TT before oven drying protected the PA of the resulting crude papain during storage at room temperature. This protection was greater than that provided by the same level of MBS (Fig. 4). Addition of MBS before drying also decreased losses of PA, but to a lesser extent than TT.

Relatively high losses of PA occurred during storage of the

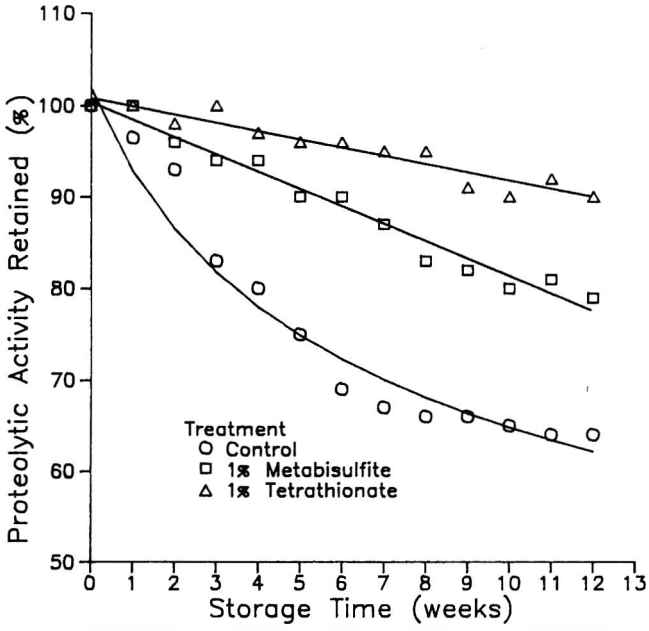

Fig. 4—Change in proteolytic activity of crude papain with or without additives during storage at room temperature. Each point = mean of triplicates. Solid lines calculated assuming empirical reaction orders of 4.5 for control and zero order for the metabisulfite and tetrathionate treated samples.

control; almost 40% of the original PA was lost in 13 wk (91 days) (Fig. 4). Castro (1981) reported 30% losses of PA for crude papain stored 75 days at room temperature. Narinesingh and Mohammed-Naraj (1988) indicated storage of sun-dried crude papain 2 wk resulted in PA losses of 24%. Since crude papain is usually exported from producing to refining countries (Leung, 1980; Flynn, 1975), it is probably stored for relatively long periods with consequent losses of PA. By adding tetrathionate to latex before drying, minimizing losses in PA may be possible, thus increasing stability of crude papain.

For the control (no additives), the decrease in PA with time was nonlinear (Fig. 4). The empirical reaction order of the PA loss over time for this crude papain, determined using the linearization procedure of Durance et al. (1986), was 4.5. Since loss of PA of crude papain is due to a number of different factors (i.e. oxidation, microbial degradation, autodigestion, interaction of protein with carbohydrates present etc.) it is expected that overall kinetics of degradation are complex.

For samples of crude papain with either additive, the decrease in PA was linear over these storage periods, which indicated a zero-order reaction. Although the color of the crude papain was not measured, no apparent change in this parameter was observed during the storage times tested.

Since a rehydrated crude papain was used in these experiments, further research using fresh papaya latex under manufacturing conditions is needed to recommend the use of TT in manufacturing papain preparations. However, TT reacts specifically with the essential SH group of the individual proteolytic enzyme papain (E.C. 3.4.22.2) (Arteaga, 1988). That fact, together with the observation that another disulfide, 2,2'dipyridyl disulfide (2PDS), increased stability of fresh papaya latex (Brocklehurst et al., 1985), suggest TT has potential application in production of crude papain.

CONCLUSION

ADDITION of TT at 1% before oven drying crude papain completely inhibited losses of PA when drying temperature was low (50–55°C). Since, in commercial practice 55°C is commonly used, addition of TT has potential industrial application. Addition of 1% MBS also protected PA during oven drying; however, its protective effect was significantly lower than that of TT. A fractional factorial experimental design showed best conditions (minimum PA losses) for drying rehydrated crude papain were: addition of 1% TT; drying at 55°C; drying load 1,190 g/m^2, and if storage prior to drying was necessary, it should be in the dark. As reported by other researchers, vacuum drying produced smallest PA losses, and in this case TT or MBS were equally effective.

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Water Mobility in Starch/Sucrose Systems: an Oxygen-17 NMR Study

P. CHINACHOTI and T. R. STENGLE

ABSTRACT

Sucrose/starch mixtures containing 0, 10, and 20% sucrose were equilibrated with water vapor enriched to 1% ^{17}O, at water activities (a_w) of 0.88, 0.93, and 0.97. In all samples, a significant quantity of water did not appear in the NMR spectrum, presumably because it was immobilized. The amount of water, which was somewhat mobile and appeared in the spectrum, increased with increasing a_w and sucrose content. The relaxation time of this NMR active water was strongly dependent on sucrose content, in a way that could not be explained by any simple model. Thus, the presence of sucrose has a profound effect on interaction of starch with water.

INTRODUCTION

WATER SORPTION STUDIES by Lang and Steinberg (1980) showed the amount of water sorbed by a sucrose-starch mixture (mechanically mixed) agreed experimentally with the value calculated by applying a mass balance equation. They therefore concluded there was no interaction between sucrose and starch which interferred with their interactions with water, and that sucrose sorbed water independently, i.e. the water was in solution at a_w above 0.86 and at 25°C (Chinachoti and Steinberg, 1984; Karel, 1973). A similar sorption study by the same group showed freeze-dried sucrose-starch mixtures sorbed less water than expected (Chinachoti and Steinberg, 1984). They speculated the process of wet-mixing and freeze-drying allowed a sucrose-starch interaction which resulted in decreased water sorbability. This was later supported by Gilbert et al.(Carillo et al., 1988, 1989) who reported that, in a freeze-dried sucrose-starch mixture, sucrose reduced the number of water binding sites on starch, as represented by the cluster model.

Those studies indicated that water sorption by a multi-component system was complicated, and such studies did not allow specific conclusions regarding molecular interactions. Attempts to study and characterize water states in heterogeneous systems from sorption isotherms may be flawed due to (1) lack of a true equilibrium, (2) plasticizing effect of water which changes structural components, and (3) lack of an appropriate mathematical expression describing the binding energies at various sites. For these reasons, other experimental approaches have been applied to obtain more dynamic information about water in foods. Nuclear Magnetic Resonance (NMR) has been very popular because of its capability to noninvasively determine water mobility by energizing a chosen nuclide, ^{1}H, ^{2}H, and ^{17}O, of water. Details of NMR use to study water are reported elsewhere (Richardson and Steinberg, 1987).

Richardson et al. (1987a, b, c) studied mobility of water in corn starch, sucrose, and a freeze-dried mixture, by applying ^{2}H and ^{17}O NMR. The relaxation rates of the bound fraction were used to calculate correlation times of 21.3 ps for starch and 100–9.5 ps (for hydration numbers of 2–21, respectively) for sucrose. Compared to the correlation time of pure water, 3.1 ps at 27°C, water in starch and sucrose was less mobile but not to the extent that it was tightly bound (Richardson et al., 1987a, b). However, we must note this conclusion pertained to the moisture in starch samples containing not more than 70% solids. Confirming the result from a study at > 70% solids would be of interest. Unfortunately, the sensitivity of the instrument drops drastically at such low water content.

For the case of a freeze-dried sucrose-starch mixture, Richardson et al. (1987c) reported a very interesting result for the heterogeneous system. Samples were hydrated to obtain 0–85% solids for a ^{17}O NMR experiment. Below 67% solids, a single Lorentzian peak was observed, and the calculated correlation time was 20.3 ps, close to that for pure starch. At solids concentrations between 67 and 83%, a composite peak was observed, which could be decomposed into two Lorentzians of the same chemical shift but different widths. This was ascribed to non-exchanging amounts of trapped water and water in the sucrose solution. Above 83% solids, a single Lorentzian peak was observed; this corresponded to 0.86 a_w, which was the saturation point for sucrose solution at 25°C. Interestingly, Chinachoti and Steinberg (1984) reported water sorption data for the same sucrose-starch system in this a_w range was almost the same as for starch alone at the same a_w. The NMR data showed the linewidth for the mixture to be the same as that for starch alone (Richardson et al., 1987c). This good agreement showed that, in a saturation situation, sucrose did not sorb water but starch did. Apparently, freeze-drying generated a supersaturated condition that did not allow complete hydration within the time frame of the study (Richardson et al., 1987c).

Based on these interesting findings that ^{17}O NMR could detect water motion in a multi-component system, our objective was to apply ^{17}O NMR to freeze-dried sucrose-starch systems in high solids concentration range, i.e. intermediate a_w (0.88–0.97), and at various sucrose levels, in order to determine the mobility and states of water at different water and sucrose contents.

MATERIALS & METHODS

Sample preparations

Native wheat starch (Manildra Milling Corp., Minneapolis, MN) and sucrose (analytical grade, Fisher Scientific, Inc., Fair Lawn, NJ) were mixed with water to obtain mixtures (60% water, 40% total solids) with 0, 10, 20, and 30% sucrose (dry basis). The slurries were freeze-dried under 3 μ Hg pressure and room temperature for 36 hr, ground, and stored in a desiccator. Their vacuum oven (60°C, 48 hr) moistures ranged from 2.0 to 3.5% dry basis. K-sorbate was added (1% dry basis) to prevent mold growth during the sorption period.

The freeze-dried starch and mixtures with sucrose were equilibrated at 25°C against saturated salt solutions of known a_w ranging from 0.88 to 0.97 (Greenspan, 1977; Stokes and Robinson, 1949). The saturated salt solutions were prepared using 1% ^{17}O enriched water (Cambridge Isotope, Cambridge, MA) and were placed on the bottom of a Proximate Equilibration Cell or PEC (Lang et al., 1981). Equilibration was considered established when weighing samples every second day showed three consecutive readings within ± 1 mg. Final moistures were calculated from weight gains.

NMR measurements

The ^{17}O spectra of starch/sucrose samples were obtained on a Varian XL-300 spectrometer operating at 40.662 MHz. Samples weighing

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Table 1—Total Moisture and NMR-Observed Moisture

Sucrose (%)	Total moisture[a]	NMR-Active moisture[b]	NMR moisture (%)	Line width (Hz)
		a_w = 0.88		
0	156	4	3	4,500
10	178	8	5	1,100
20	205	39	19	840
		a_w = 0.93		
0	241	60	25	3,200
10	264	111	42	560
20	312	225	72	480
		a_w = 0.97		
0	300	102	34	2,900
10	483	257	53	400
20	679	465	68	330

[a] mg water/g dry solids
[b] NMR-active moisture = mg water/g dry solids that contributed to NMR signal.

between 100 and 500 mg were packed in the bottom of 10 mm NMR tubes and placed in the probe such that the entire sample was in the active region of the receiver coil. A 90° pulse width and a spectral width of 100 KHz were used. Receiver deadtimes ranged from 50 to 200 μs. An acquisition time of 25 ms was more than sufficient to collect data. In most cases, a satisfactory spectrum could be obtained in 1–4 hr. Due to the broad lines and the small amounts of observable ^{17}O in the samples, the signal to noise ratio was never high. It was as low as 4 in some samples. In all cases, the spectrum consisted of a single line, width varying from several hundred to several thousand Hz. Linewidths were reported as the width in Hz at half height, which could be converted to the transverse relaxation time, T_2, if a Lorentzian shape function is assumed. Since there was some evidence that the shape was not a pure Lorentzian (vide infra), linewidths rather than T_2 values were reported.

The total intensity of the observed ^{17}O signal was determined by integration. A special problem arose with powdered samples, where packing density (i.e. filling factor) could affect signal strength. To avoid this, small samples (100 to 500 mg) were used such that the entire sample, regardless of bulk density, contributed to the NMR signal. Thus, all the ^{17}O nuclei in the sample contributed equally to the observed signal, so signal intensity was directly proportional to total weight of mobile water. Signal intensities were calibrated by integrating the signal from a sample of *ca.* 100 mg pure water containing ^{17}O at its natural level. This was verified by measuring the signal integral from a series of water samples of 100 to 1100 mg. The relationship between weight and integral value was linear (correlation coefficient = 0.9999) up to 630 mg water. An unavoidable problem was caused by decay of signal strength during receiver deadtime. Experimental intensities were corrected to zero deadtime by assuming an exponential decay with time constant T_2, where T_2 was estimated from linewidth. This is subject to error, but we found it the most effective way to obtain intensities, given the wide range of linewidths and intensities of our samples. A comparison of intensity data with known moisture of samples allowed calculation of weight of NMR-active moisture, and the fraction of total moisture which contributed to the NMR signal.

RESULTS & DISCUSSION

CLEARLY, a large fraction of the water present was not visible in these spectra obtained with a high resolution spectrometer. The experimental data are presented in Table 1. The NMR-active moisture was the weight of water calculated from the intensity of the NMR signal. Linewidth of the NMR signal was observed to vary greatly with sucrose content; a small amount of sucrose produced a marked narrowing of the line. This is illustrated in Fig. 1.

The striking point about the linewidth was the effect of relatively small amounts of sucrose. For example, at a_w = 0.88, addition of 10% sucrose caused total moisture to increase by 14%, but linewidth was reduced by a factor of four. It is difficult to explain this effect if we assume all water in the sample contributed to the NMR signal. It was not possible to fit these data to a two-site fast-exchange model. Furthermore, we observed a large increase in signal to noise ratio on addition of sucrose, which could not be explained entirely by change

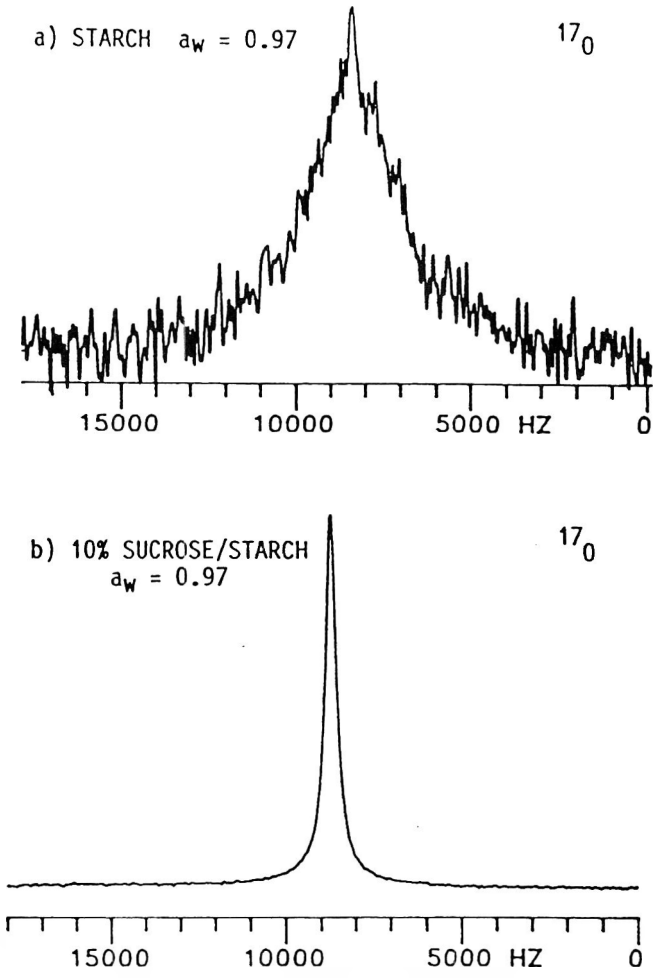

Fig. 1—^{17}O NMR spectra of starch and 10% sucrose/starch mixture equilibrated at a_w=0.97; equilibration and spectrometer both 25°C. The scale is presented to give estimate of linewidth; it does not indicate absolute chemical shift. (a) Pure starch, sample weight = 355 mg, number of scans collected = 91,400; (b) 10% sucrose/starch mixture, sample weight = 277 mg, number of scans = 56,700.

in linewidth. Thus we hypothesized that only a fraction of the total water was observed in the NMR spectrum. This point was confirmed by quantitative intensity measurements.

The intensity data showed that only a small percentage of total moisture was observed in samples of pure starch. When 10% sucrose was added, NMR active moisture almost doubled, and linewidth decreased by a factor ranging from 4 to 7 (Table 1). Additional sucrose caused small reductions in linewidth, along with increases in the quantity of water observed. This explained the large apparant increase in signal-to-noise ratio on adding sucrose. The signal to noise ratios were not measured quantitatively, so they were not tabulated here.

Results showed a significant fraction of the water in the starch/sucrose systems was bound sufficiently tightly to be immobile on the NMR time scale. The remaining water was somewhat more mobile, depending on composition of the carbohydrate phase. The effect of a_w was quite major. In pure starch, a comparison of total moisture with NMR-active moisture (Table 1) showed the amount of tightly bound water increased slowly with a_w, while the quantity of NMR-active water increased rapidly. This was consistent with a model in which there is an upper limit on the amount of water that can be tightly bound. The additional water absorbed at high a_w entered a mobile phase. The mobility of this phase increased only slowly with a_w, as shown by the slow decrease in linewidth.

Note that there was no evidence in the NMR data for exchange of water between the two phases.

The effect of added sucrose on absorbed water could not be explained by any simple model. The data showed adding 10% sucrose caused a drastic decrease in linewidth, accompanied by almost doubling in quantity of NMR-active water. The simple fast-exchange, two-site model is often used to explain such behavior (Bovey et al., 1988). For example, at a_w = 0.88 and 10% sucrose, we might assume the water existed in two domains of different mobilities (and hence different linewidths). The less mobile domain would contain 4 mg water with mobility corresponding to 4500 Hz linewidth, i.e. the same as in pure starch. The other domain also contained 4 mg water but with greater mobility. Reasonable mobility for this fraction might be that of pure water, linewidth 63 Hz. The experimental observed linewidth would be the average of the two. It is clearly impossible to obtain agreement between the observed value of 1100 Hz and such a model. One modification of the model, which would give agreement with observed values, would be to assume the addition of 10% sucrose affected the less mobile domain such as to reduce linewidth from 4500 to 2150 Hz.

The amounts of NMR-active and tightly bound water (i.e. immobile and therefore NMR-inactive) can be obtained from Table 1. These quantities give insight into the effect of adding sucrose to starch. We consider the total moisture divided into two independent fractions, NMR-active and tightly bound. For the three samples at a_w = 0.88, we find the quantity of tightly bound water remained almost constant (within experimental error) as sucrose was increased. Thus, sucrose had little effect on bound water, but it did affect the mobile fraction. The mobility of water increased as additional water was accommodated by the added sucrose. Analysis in terms of a fast-exchange, two-state model required that the relaxation time at the less mobile site must increase with additional sucrose, as discussed above.

A similar pattern was seen for the samples equilibrated at a_w = 0.97. Here the quantity of tightly bound water was more or less independent of added sucrose. The amount of NMR-active water increased greatly as sucrose was added, and, hence, the overall moisture content was increased. The linewidth decreased in a fashion similar to the a_w = 0.88 samples. The a_w = 0.93 samples showed a quite different and puzzling behavior. Here the quantity of tightly bound water decreased to roughly half the value as 20% sucrose was added. Consequently, the NMR-active water increased more than the overall increase in moisture content as sucrose was added. This effect could be explained by a mechanism wherein added sucrose caused release of water tightly bound to starch. This is somewhat implausible in view of the contrasting behavior at a_w = 0.88 and 0.97. This anomaly may have been caused by experimental problems. This possibility is being investigated further.

The accuracy of water quantity measurement was affected by several variables. The reproducibility of dry sample equilibration with water vapor must be considered. Only small quantities of ^{17}O-enriched water could be used in preparing eaach sample, because of cost. Consequently, sophisticated procedures to ensure true equilibration could not be used. Integration of NMR signals is subject to error caused by poor signal/noise. The deadtime correction was open to question, especially considering somewhat different results were obtained at different deadtimes (which implied a non-Lorentzian line shape). Finally, there was some evidence that there were changes in NMR signal as a sample aged. This aging effect is being studied further. Considering these uncertainties in measurement, we must consider our data to be semi-quantitative at best. In worst possible cases, values of NMR-active moisture could be in error by a factor of two. Nevertheless, we feel that trends shown are reliable. The linewidths are more reliable than integrated intensities; the signal to noise ratio determined the reproducibility, which varied from 5% to 20% in the worst cases.

Our results did not lead to conclusive answers concerning starch-sucrose-water interactions. Nevertheless, some conclusions can be drawn. We found that determination of signal intensities and calculation of NMR-active water were of crucial importance. Presumably, this would also be true for other NMR investigations of water in foods. Such studies have not been made in the past. Even with this additional measurement, it was not always possible to interpret NMR data in an unambiguous way. Although our linewidth data for the NMR-active fraction could be explained in terms of a modified two-site, fast-exchange model, we must assume a different linewidth for the less mobile component in each sample. This would leave too many adjustable parameters. A definitive result would require additional experiments and refinements, such as solid state ^{17}O spectra, study of the frequency relationships of linewidths, and improved accuracy of signal intensity determination.

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Effects of Succinylation on β-Lactoglobulin Foaming Properties

L.G. PHILLIPS and J.E. KINSELLA

ABSTRACT

The extent of succinylation of β-lactoglobulin (β-Lg) increased logarithmically with increasing concentrations of succinic anhydride. The surface pressure of 27.5% succinylated β-Lg was higher than native β-Lg but higher levels of succinylation (50% and 100%) reduced the surface pressure. Overrun and foam stability were reduced following succinylation. The electrostatic interaction caused by the addition of 100% succinylated β-lactoglobulin (0.5g/100 mL) to a solution (2.5%) of native β-lactoglobulin at pH 4.0 improved overrun (47%) and foam stability (61%).

INTRODUCTION

PROTEINS are key components in many food foams ie. breads, cakes, meringues and whipped toppings. Proteins are amphipathic and many are surface active with the potential to quickly move to an air/water interface, reorient and form stable films (Graham and Phillips, 1976; Kinsella and Phillips, 1989; German and Phillips, 1989). Many studies have investigated the foaming properties of whey proteins (Kinsella and Whitehead, 1989; Kinsella and Phillips, 1989). Whey protein preparations have variable foaming properties. The predominant protein in whey, β-lactoglobulin (β-Lg) is very surface active (Waniska and Kinsella, 1985) and produces foams which have higher overruns than egg white (Phillips, 1988). Therefore, much of the work involving whey has centered around determining why there is such a disparity between the foaming properties of whey and β-lactoglobulin.

Certain components found in whey decrease foam formation and/or foam stability. These include lipoproteins, calcium and lactose (Kinsella, 1982; Kinsella and Whitehead, 1989; Maubois et al., 1987; Morr, 1985; Phillips, 1988; Phillips et al., 1989a). The manner in which whey is processed can also affect the foaming properties (Mangino et al., 1987) eg. heating whey protein isolate solution (pH 7.0) for 10 minutes at 55°C significantly improved foaming (Phillips et al., 1990b). Historically charge repulsion has been considered the predominant contribution of electrostatic interactions imparting foam stability by keeping adjacent bubbles from coalescing (Halling 1981, Kinsella, 1982). There are some contradictions to this generalization. For example, whipping whey protein isolate at values close to the isoelectric point enhances electrostatic attraction, improves foam formation and enhances foam stability (Phillips et al., 1990b). Also, adding the basic protein, lysozyme to whey solutions (pH 8.0) improves foam stability with the resultant foams superior to egg white even when subjected to heat (Phillips et al., 1989b). The enhanced foaming properties of this system have been attributed to complex formation resulting from electrostatic attractions between the negatively charged whey protein and the positively charged lysozyme (Poole et al., 1984; Phillips et al., 1989b).

One approach for determining the importance of electrostatic interactions involves modifying the protein such that charge repulsion or attraction is altered and then monitoring the effects on foaming properties. Modification of the ϵ-amino groups of lysyl residues in proteins by acylation eg. succinylation increases the negative charge on the protein (Means and Feeney, 1971). Modification of the lysyl residues in proteins by acylation has been used to alter functional properties (Kinsella and Shetty, 1979; Brinegar and Kinsella, 1980; Richardson and Kester, 1984; Richardson, 1985). Kim and Kinsella (1987) used succinylation to improve the foaming properties of soy proteins.

The objectives of this study were to determine the effects of altering the negative charge of β-Lg on foaming properties at pH 7.0 and to determine the effects of adding succinylated β-Lg to native β-Lg on foaming at pH 4.0 and 8.0.

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MATERIALS & METHODS

Protein

The studies were conducted with β-lactoglobulin purified from whey protein isolate prepared by ion exchange chromatography (Mitchelstown Isolates, Cork, Ireland) using the method of Aschaffenburg and Drewery (1957).

Succinylation of β-lactoglobulin

Succinylation was achieved by adding succinic anhydride in small increments (0.05 g) to 100 mL of β-Lg (0.8g/100g) dissolved in 0.1M phosphate buffer (pH 8.0) until a desired level of succinylation was obtained (Franzen and Kinsella, 1976). The pH of the solution was maintained by the addition of 3 N NaOH. After the addition of succinic anhydride the reaction mixture was stirred until the pH stabilized. The solution was dialyzed against distilled water at 4°C to remove excess succinic acid and lyophilized. The extent of succinylation of β-Lg was determined with TNBS (trinitrobenzenesulfonate)(Kim and Kinsella, 1986).

Turbidity

The aggregation of protein dispersions was determined by transmittance at 500 nm using a Spectronic 700 spectrometer (Bausch and Lomb, Rochester, NY). The values were expressed as Tb = (1 − T500) × 100. A value of 100 corresponded to a completely turbid solution (0 transmittance).

Surface tension

To determine the interfacial characteristics of the different β-Lg solutions, surface tension was measured using a Fisher tensiomat with a du Nuoy ring (Fisher Scientific, Springfield, NJ). Surface tension of the distilled water used in protein solubilization was measured. The surface tension of the various protein solutions was measured after hydration (30 min). Changes in temperature and instrument accuracy were accounted for using the surface tension of water at 25°C (72.14 mN/M) and the following equations:

$$\text{Correction factor} = 1/[1 - (72.14 - \text{measured surface tension of water})]$$

$$\text{Corrected surface tension} = \text{Actual value} \times \text{Correction factor}$$

$$\text{Surface Pressure (mN/M)} = 72.14 - \text{Corrected surface tension}$$

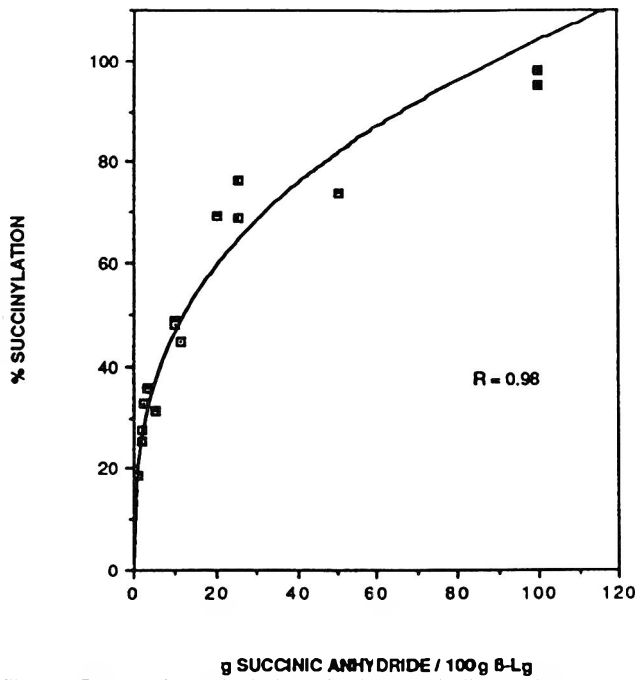

Fig. 1—Extent of succinylation of β-lactoglobulin at pH 8.0 as a function of total succinic anhydride added. The data fit the following equation: Log Y = Log 21.1142 + (0.3466) Log X.

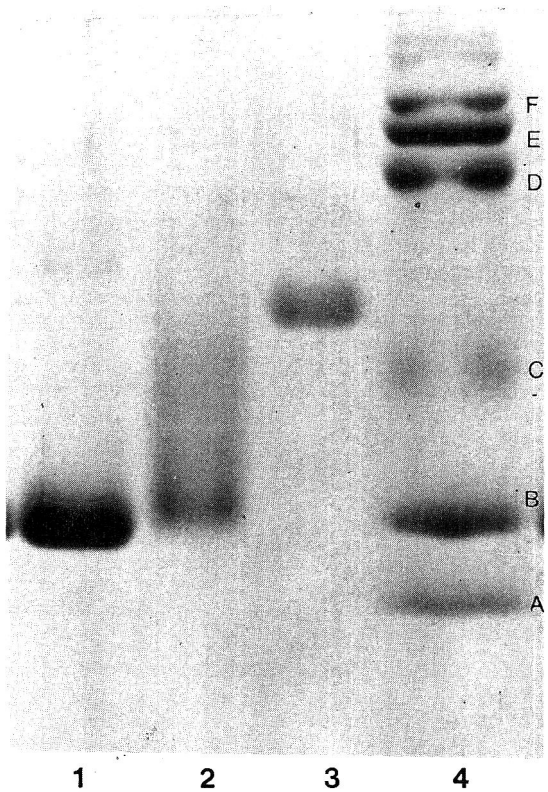

Fig. 2—SDS-PAGE gel electrophoretogram of 1) β-Lg(27.5S), 2) β-Lg(50S), 3) β-Lg(100S) and 4) standard: α-lactalbumin (a), β-lactoglobulin (b), immunoglobulin G (light chain) (c), bovine serum albumin (d), immunoglobulin G (heavy chain) (e) and lactoferrin (f).

Foaming properties

The foaming properties of the native and modified β-Lg solutions were measured by measuring overrun using the method of Phillips et al. (1987, 1990a). Overrun was determined (3 replicates) using 75 mL of the modified β-Lg solutions (2.5 g/100 mL) solubilized for 30 min with adjustment to the appropriate pH using either 0.1N HCl or 0.1N NaOH as needed. The mixtures were whipped for 5 min intervals a total of 20 min using a Sunbeam Mixmaster mixer (Sunbeam Corporation, Oak Brook, IL). The amount of air incorporated was recorded as percent overrun.

Foam stability was measured (3 replicates) after whipping 15 min as described by Phillips et al. (1987, 1990a). The weight of the liquid separating from the foam was continuously recorded using a Sartorius balance (model 1212MP Brinkman Instruments Co., Westbury, NY) connected to an Apple IIe computer using an interface board (IMI State College, PA). The time required for half the original weight of the foam after whipping to drain as liquid was reported as 50% drainage, an index of instability. The drained liquid was collected in a tared container on the balance pan.

Table 1—Effects of succinylation on rate of overrun development of β-lactoglobulin at pH 7.0[a]

Succinylation (%)	Surface pressure (mN/M)	SD	Rate of foam development (△% Overrun/△ Min)	R
0.0	13.9	0.55	29.98	0.9848
27.5	18.4	1.59	0.0	0.0
50.0	13.6	1.21	13.92	0.9827
100.0	14.3	0.25	11.40	0.9889

[a] R = correlation coefficient; SD = standard deviation; Reported means are average of four replicates.

Electrophoresis

The succinylated β-Lg samples were analyzed by the sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method of Laemmli (1970). A Hoefer SE200 miniature slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) was used. Bovine serum albumin, β-lactoglobulin, α-lactalbumin, lactoferrin and immunoglobulin G (Sigma, St. Louis, MO) were used as standards.

Statistics

Data were analyzed using the statistical analysis package, release 82.3, computer program with the general linear models option (SAS, 1985). The mean square error term from the analysis of variance was used to calculate 95% confidence intervals. Overrun development was determined from the slope measured by linear regression of the individual overrun curves. The Pearson correlation coefficient (R) was recorded for each, as a measure of linearity.

RESULTS & DISCUSSION

Effects of succinylation on β-lactoglobulin foams

The extent of succinylation of β-Lg increased logarithmically with increasing amounts of succinic anhydride added (Fig. 1). This was comparable to the results obtained for succinylation of other proteins where 100% succinylation was achieved using relatively low ratios of succinic anhydride to protein (Means and Feeney, 1971; Franzen and Kinsella, 1976; Kinsella and Shetty, 1979; Kim and Kinsella, 1986). The results suggest that the lysine residues of β-Lg are relatively accessible and reactive.

SDS-PAGE was conducted after succinylation (Fig. 2). There was no measurable change in mobility for 27.5% succinylated β-Lg (β-Lg(27.5S)) compared to native β-Lg; a heterogeneous mixture of modified proteins was observed for 50% succinylated β-Lg (β-Lg(50S)), while a single band was observed for 100% succinylated β-Lg (β-Lg(100S)).

The effects of succinylation on surface pressure (π) were studied. The π of β-Lg (27.5S) increased by 32% compared to the unmodified β-Lg ($p<0.05$) (Table 1). As the extent of succinylation of β-Lg was increased to 50 and 100%, the π returned to levels close to the π of unmodified β-Lg (Table 1). This was similar to results from the progressive succinylation of glycinin (Kim and Kinsella, 1987).

The increased π for β-Lg(27.5S) may reflect greater spreading by the unfolded β-Lg and/or an increased interaction be-

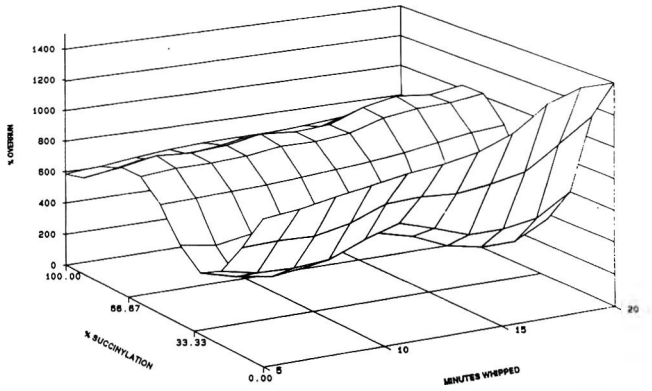

Fig. 3—Effects of extent of succinylation (0, 27.5, 50.0 and 100.0%) on overrun of β-lactoglobulin at pH 7.0. The succinylated β-lactoglobulin (2.5g/100 mL) was whipped for 5 minute intervals.

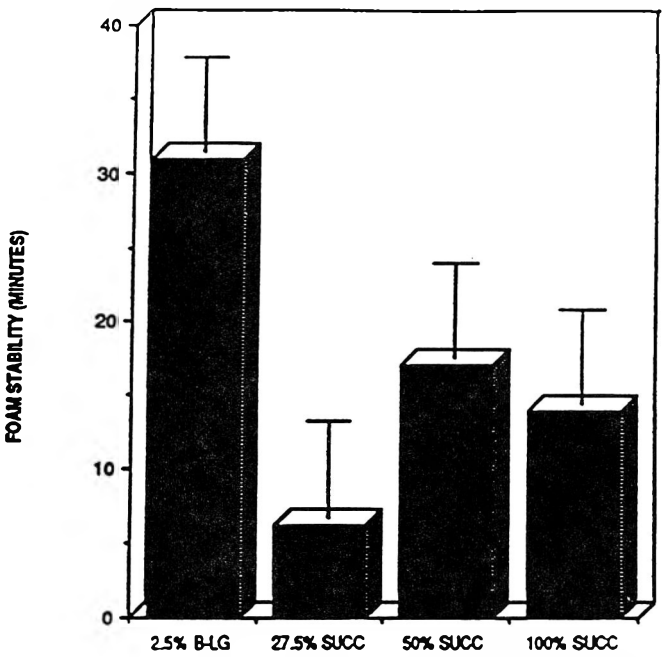

Fig. 4—Effects of extent of succinylation (0, 27.5, 50.0 and 100.0%) on foam stability of β-lactoglobulin at pH 7.0 (with 95% confidence intervals). Succinylated β-lactoglobulin (2.5g/100 mL) was whipped 15 min and foam stability measured.

tween the protein at the air/water interface. This could be caused by partial unfolding allowing for reorientation and better interaction with the interface. The increased unfolding of proteins following succinylation was shown by Kinsella and Shetty (1979). The observed reduction in π with further succinylation may occur because of the increase in negative succinyl groups on the protein causing electrostatic repulsions which inhibited protein/protein interactions at the interface.

The foam overrun was reduced by succinylation of β-Lg (Fig. 3). Although for 27.5% succinylation π increased at the air/water interface (Table 1). Apparently protein/protein interactions were impaired by the increased net negative charge with subsequent reduced air incorporation as demonstrated by a 70% reduction in overrun after 20 minutes whipping for β-Lg(27.5S) (Fig. 3). The overrun after 20 minutes whipping was reduced by 29% and 40% for β-Lg(50S) and β-Lg(100S) respectively compared to the unmodified β-Lg (Fig. 3).

The rate of foam development or change in overrun with time, which reflects how well the protein solution incorporates air into the foam with continued whipping, was also reduced by succinylation (Table 1). The β-Lg(27.5S) solution did not incorporate additional air into the foam after 5 min whipping (Table 1) whereas relatively more air was being continually incorporated into the foam with β-Lg(50S) and β-Lg(100S) but not as efficiently as with the native β-Lg.

Foam stability was also adversely affected by succinylation. The greatest change in foam stability was observed with β-Lg(27.5S)(Fig. 4), i.e. 80, 45 and 55% reduction in foam stability respectively, for the three levels of succinylation (27.5, 50 and 100%). The effects of succinylation on overrun and foam stability were highly correlated ($R = 0.9580$). These correlation data suggest a common mechanism.

One possible explanation for reduction in foaming with succinylation of β-Lg is suggested by the SDS-PAGE (Fig. 2). Conceivably at 27.5% succinylation the succinic anhydride may have reacted predominantly with lysines on the surface of β-Lg allowing it to retain some globular conformation but with a highly electronegative surface charge which did not improve foam formation during whipping. A range of molecular species (sizes) was observed for β-Lg(50S) (Figure 2) suggesting either some type of polymerization or differences in accessibility of lysine residues of β-Lg to succinic anhydride leading to charged species with different affinities for SDS. If a distribution of charged molecules were obtained for β-Lg(50S), this may have resulted in the observed populations of β-Lg with different degrees of electronegativity. The lysine residues inside the protein may initially have been slightly less accessible until repulsive forces induced by further succinylation caused unfolding which made these interior lysine residues available (Kim and Kinsella, 1987; Kinsella and Shetty, 1979). Further succinylation may lead to some type of polymerization which was not disrupted by the reducing agents used for SDS-PAGE. Some species may have been more unfolded than others resulting in improved protein/protein interaction and film formation. Brinegar and Kinsella (1981) reported polymerization of β-Lg following citroconylation but this was via disulfide bond formation. The β-Lg(50S) had the most observed heterogeneity (Fig. 4) and yielded the most stable foams with the highest overrun values of all succinylated samples (Fig. 3 and 4).

Effects on foaming properties of adding 100% succinylated β-Lg to native β-Lg

Providing conditions where electrostatic attractions between proteins are enhanced can improve foam stability (Poole et al., 1984, Phillips et al., 1990b; 1989b). Succinylation of β-Lg imparts a higher negative charge and affects foaming proper-

Table 2—Effects of adding 100% succinylated β-lactoglobulin on turbidity and rate of overrun development of β-lactoglobulin[a]

Protein	pH	Tb	SD	Surface pressure (mN/M)	SD	Rate of foam development (△% Overrun/△ Min)	R
2.5% β-Lg	8.0	2.0	0.006	17.8	0 81	37.09	0.9538
2.5% β-Lg + 0.5% β-Lg(100S)	8.0	2.8	0.008	18.6	0 78	46.64	0.9366
2.5% β-Lg	4.0	20.3	0.033	18.8	1 65	4.14	0.9903
2.5% β-Lg + 0.5% β-Lg (100S)	4.0	99.9	0.001	16.7	1 06	18.90	0.9730

[a] Values are expressed as $Tb = (1 - T_{500}) \times 100$. A value of 100 would be a completely turbid solution (0 transmittance). SD = standard deviation. R = correlation coefficient. Reported means are average of four replicates.

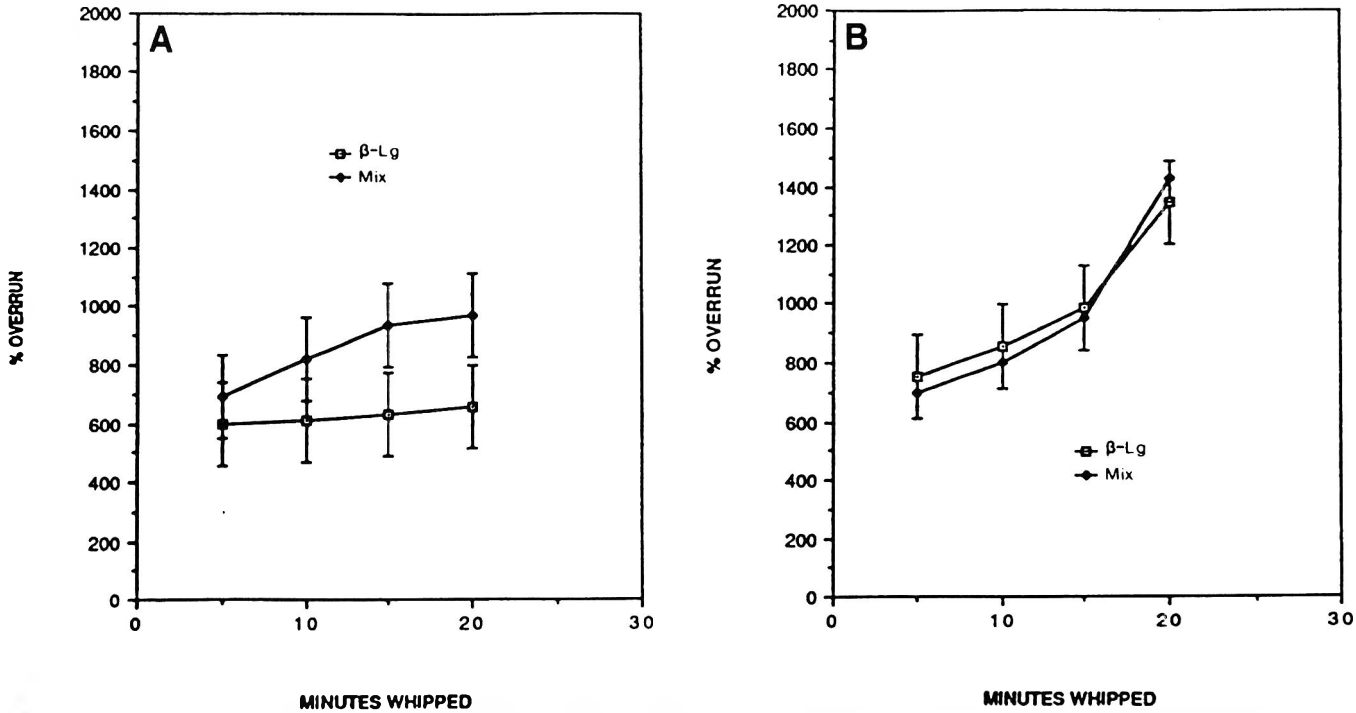

Fig. 5–Effects of adding 100% succinylated β-lactoglobulin (0.5g/100 mL) on overrun of β-lactoglobulin solutions (2.5g/100 mL) (with 95% confidence intervals). (A) pH 4.0; (B) pH 8.0.

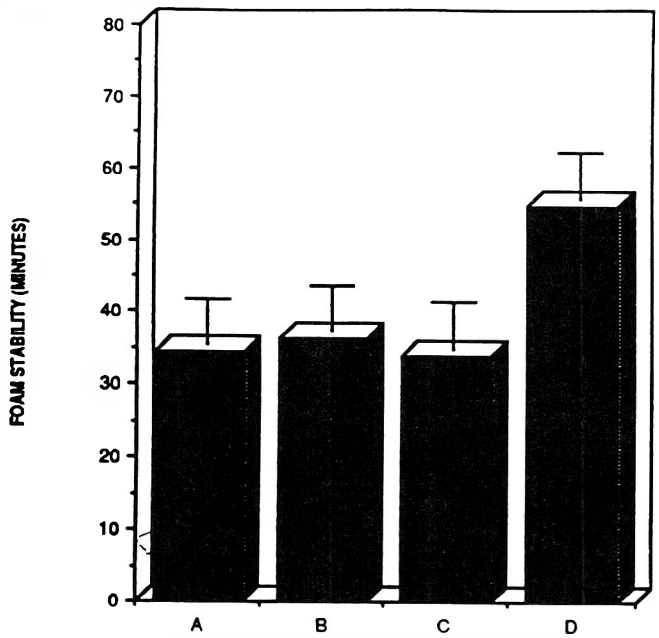

Fig. 6–Effects of adding 100% succinylated β-lactoglobulin (0.5g/100 mL) on foam stability of β-lactoglobulin solutions (2.5g/100 mL) (with 95% confidence intervals). (A) β-lactoglobulin (pH 8.0); (B) β-lactoglobulin (pH 8.0) with β-lactoglobulin (100% succinylated) (0.5g/100 mL); (C) β-lactoglobulin (pH 4.0); and (D) β-lactoglobulin (pH 4.0) with β-lactoglobulin (100% succinylated) (0.5g/100 mL).

ties. Therefore, the interactions of β-Lg(100S) with unmodified β-Lg at pH 4.0 and pH 8.0 were studied.

At pH 8.0, the turbidity of the β-Lg solution was changed only slightly by adding the β-Lg(100S) (Table 2). At pH 4.0, adding 0.5% β-Lg(100S) to a 2.5% β-Lg solution resulted in an increase (392%) in turbidity (Table 2) although the measured π values were slightly higher than for native β-Lg (Table 2). The addition of β-Lg(100S) to unmodified β-Lg caused a slight reduction in π (11%) at pH 4.0 (Table 2).

The addition of β-Lg(100S) caused a 47% increase in overrun at pH 4.0 (Fig. 5a) whereas at pH 8.0 there was no change in overrun when the succinylated β-Lg was added (Fig. 5b). The addition of completely succinylated β-Lg affected the rate of overrun development differently at different pH values. At pH 4.0 the unmodified β-Lg foams demonstrated a slight increase in air incorporation with time. Adding completely succinylated β-Lg (0.5g/100mL) increased the rate of foam development by 357% (Table 2). The rate of foam development at pH 8.0 was enhanced slightly (26%) compared to β-Lg alone. The stability of foams formed at pH 4.0 was increased by 61% (Fig. 6) whereas at pH 8.0 the foam stability was not significantly increased by adding the succinylated β-Lg (Fig. 6).

The greatest changes for all parameters occurred at pH 4.0 because the many negative carboxyl groups added via succinylation increased the negative charge on the acylated β-Lg. Adding succinylated β-Lg(100S) to the unmodified β-Lg at pH below the pI of native β-Lg (5.20) resulted in electrostatic attraction between the two. This interaction was exhibited by increases in turbidity, overrun and foam stability at pH 4.0.

In conclusion, these experiments demonstrated involvement of electrostatic interactions during film formation. Succinylation of β-Lg increased the negative charge on the protein. Foams made with succinylated β-Lg were unstable presumably because of excess intermolecular charge repulsion which prevented formation of cohesive films. The mixing of succinylated β-Lg(100S) with native β-Lg could improve foam stability at pH 4.0. Differences in charge between the more negative succinylated β-Lg and the more positive native β-Lg allowed for electrostatic attraction as demonstrated by an increase in turbidity and improved foaming properties.

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A Research Note

Effect of Irradiation on Total Glycoalkaloids in Kennebec and Russet Burbank Potatoes

N. I. MONDY and K. SEETHARAMAN

ABSTRACT

Effect of irradiation and storage conditions following irradiation on total glycoalkaloids (TGA) of Russet Burbank and Kennebec potatoes stored 4 and 12 wk was investigated. TGA increased significantly ($p < 0.005$) immediately following irradiation in both cultivars, but decreased upon storage. TGA was higher in Kennebec than Russet Burbank often exceeding levels considered safe for human consumption. At 5°C TGA was higher in tubers stored in paper as compared to polyethylene, while at 20°C this was reversed.

INTRODUCTION

SPROUTING of potatoes is undesirable since it results in shrivelling, increased susceptibility to bruise, and build-up of naturally occurring toxic compounds, glycoalkaloids. Common methods of sprout inhibition are cold storage, application of chemical inhibitors or irradiation. An irradiation dose of 10 krads was sufficient to prevent sprouting of potatoes irrespective of variety and storage temperature (Thomas, 1984).

Glycoalkaloids are naturally occurring toxicants present in potatoes and have been shown to play a role in the disease and pest resistance mechanism of the plant. Tubers containing total glycoalkaloids (TGA) at levels higher than 20 mg/100g fresh weight are considered toxic (Morris and Lee, 1984) and unfit for human consumption. Glycoalkaloids have anticholinesterase activity (Patil et al., 1972), and high levels of consumption by humans has resulted in severe illness and occasional death (McMillan and Thompson, 1979).

Observations in our laboratory over several years have shown Kennebec to be higher in TGA than other cultivars. Examination of the data presented by Sinden and Webb (1972) and Fitzpatrick et al. (1977) also showed Kennebec cultivar to be higher in TGA as compared to other cultivars. Patil et al. (1971a) reported this cultivar to be more susceptible to higher increases in TGA, on exposure to light, as compared to 20 other cultivars.

Researchers have differed in their findings concerning the effect of irradiation on TGA of tubers. Patil et al. (1971b) observed no significant changes in glycoalkaloid content of gamma irradiated tubers when exposed to light for five days following irradiation. Wu and Salunkhe (1977a) reported an 11–79% inhibition in TGA formation when wounded tubers were treated with gamma irradiation at 25–100 krads. Bergers (1981) found no effect of gamma irradiation at 10, 50 and 300 krads on TGA of potatoes when stored in the dark over 4 months at 10°C. On the other hand, Nair et al. (1981) reported 70% inhibition of glycoalkaloid synthesis using a 10 krad dose.

Sprouts contain high levels of TGA, and possibly inhibition of sprout growth, either by irradiation or by chemical means may result in inhibition of TGA synthesis. Mondy and Ponnampalam (1985) reported inhibition of TGA formation using isopropyl-N(3-chlorophenyl)-carbamate (CIPC). Wu and Salunkhe (1977b) found CIPC effective in controlling wound induced TGA formation, although they did not observe any effect on existing TGA of the tubers. Mondy et al. (1978) observed inhibition of TGA synthesis when potato plants were sprayed with maleic hydrazide.

The packaging materials used in storing potatoes affected their TGA. Gosselin and Mondy (1989) stored potatoes at 20°C and reported higher levels of TGA in tubers packaged in polyethylene as compared to those stored in mesh or paper. Gull and Isenberg (1960), and Wu and Salunkhe (1975) attributed differences in TGA to the amount of light transmitted through the packaging materials. No studies have been reported relating TGA of irradiated potatoes to packaging materials used following irradiation.

The objective of our study was to investigate the effect of irradiation on TGA content of potatoes and to study the effect of packaging materials on TGA of irradiated potatoes.

MATERIALS & METHODS

Russet Burbank and Kennebec potatoes grown at Cornell Vegetable Research Farm at Freeville, NY were used. Potatoes were harvested 22 wk after planting and stored 4 wk in the dark at 5°C (95% RH) prior to analysis.

Potatoes were irradiated using a ^{60}Co source. The dose rate of the source was measured with a Reuter-Stokes ionization chamber calibrated with a National Bureau of Standards secondary source.

Russet Burbank potatoes were irradiated at 10 and 30 krads and packaged in either kraft paper bags with mesh window, or in 2 mil polyethylene bags (10 lb) with forty-eight 1/4" holes, about 3" apart and stored at 5°C and 20°C. Polyethylene bags were perforated to simulate conditions commonly used in the market. Perforations were necessary to prevent mold and decay formation in the tubers. The Kennebec tubers were irradiated at 10 and 100 krads and were packaged in kraft paper bags and stored at 5°C.

Unirradiated controls and irradiated tubers of both cultivars were analyzed for TGA immediately following irradiation, and after 4 and 12 wk at either 5°C or 20°C. Unirradiated tubers of both cultivars were stored in a manner similar to irradiated tubers and served as controls.

Tubers were cut longitudinally from bud to stem end in order to include both the apical and basal sections. Slices were subsequently separated into cortex and pith tissues along the vascular ring, frozen, lyophilized in a Stokes freeze dryer, ground in a Wiley mill through a 40-mesh screen and stored under nitrogen until analyzed. The cortex tissue, along with the periderm, was used to analyze TGA.

Total glycoalkaloids

TGA was determined using the method described by Mondy and Ponnampalam (1983) on the cortex section of the tuber, since this is the section known to be the highest in TGA. Four determinations were made on the control and irradiated tubers.

Statistical analysis

Statistical significance of data was determined using analysis of variance with protected LSD test (Steel and Torrie, 1980).

RESULTS & DISCUSSION

In both cultivars TGA of the tubers increased significantly ($p < 0.005$) immediately following irradiation (Fig. 1). TGA was

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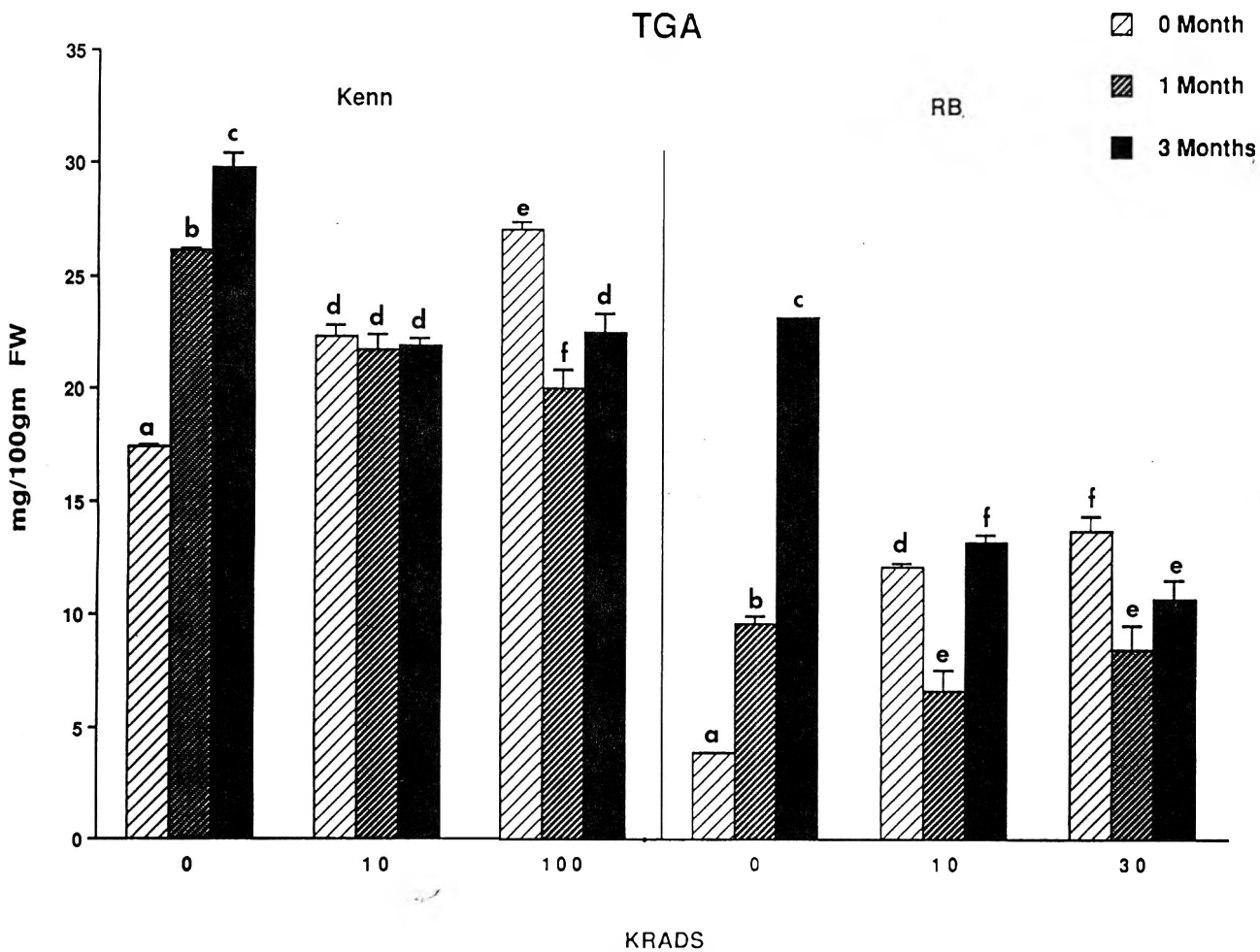

Fig. 1—Effect of storage time at 5°C on total glycoalkaloids (TGA) of Kennebec (Kenn) and Russet Burbank (RB) potatoes treated with different doses of irradiation. Bars with different letters are significant at $p < 0.01$. Means of four determinations.

higher in tubers treated with 30 and 100 krads as compared to 10 krads in the cultivars.

Following storage TGA decreased ($p < 0.01$) in both cultivars of irradiated tubers as compared to levels present immediately following irradiation. However, in the unirradiated control tubers TGA increased during storage (Fig. 1). Several researchers found that TGA of unirradiated tubers increased following storage (Zitnak, 1953; Cronk et al., 1974; Mondy and Ponnampalam, 1985; Munshi and Mondy, 1988).

At all levels of treatment the TGA of Kennebec was higher and the content was above levels considered safe for human consumption.

In the RB cultivar, a significant interaction ($p < 0.05$) was found between irradiation dose, storage temperature and packaging materials. Effect of packaging material on TGA was greatly influenced by storage temperature. Tubers stored at 5°C in paper had a higher TGA ($p < 0.005$) than tubers stored in polyethylene. However, at the higher temperature (20°C), the opposite effect of packaging materials was observed, i.e. TGA was higher ($p < 0.005$) in tubers packaged in polyethylene than in paper (Fig. 2). Gosselin and Mondy (1989) also reported higher TGA in tubers stored in polyethylene bags at 20°C as compared to tubers stored in mesh or paper.

TGA of irradiated tubers decreased following one month storage and then increased after 3 months storage. This trend was observed in both cultivars. Bergers (1981) did not find any effect of irradiation on TGA of potatoes stored up to four months at 10°C. However, his conditions of storage and irradiation dose rate differed greatly from those used in our study. Mathur (1963) reported a higher dose rate was more effective in controlling sprouting than a lower dose rate. Therefore, possibly the very high dose rate, 2.2 krads/min, used by Bergers (1981) completely inhibited TGA synthesis resulting in no differences in TGA over storage. On the other hand, our study used a lower dose rate (0.12 krads/min), and possibly resulted in less drastic effect on the TGA synthesis mechanism.

Jadhav et al. (1973) found alanine, leucine and arginine were utilized in TGA synthesis and Ussuf and Nair (1972) reported an increase in those amino acids 24 hours following irradiation. Therefore, the large increase in TGA observed immediately following irradiation may have resulted from increased synthesis following production of amino acids during irradiation. The subsequent decrease following storage may have resulted from reduced TGA synthesis.

In our study, irradiation inhibited TGA synthesis during storage, although this effect did not seem to be permanent. A dose of 10 krads, the optimum dose for sprout inhibition, was most effective in lowering TGA of the tubers. TGA levels of Kennebec tubers were close to those considered unsafe for human consumption (20 mg/100g). Varietal differences must be considered when irradiating potatoes. When storing irradiated potatoes at room temperature (20°C) paper was the preferable packaging material, but for storing at 5°C polyethylene was preferable.

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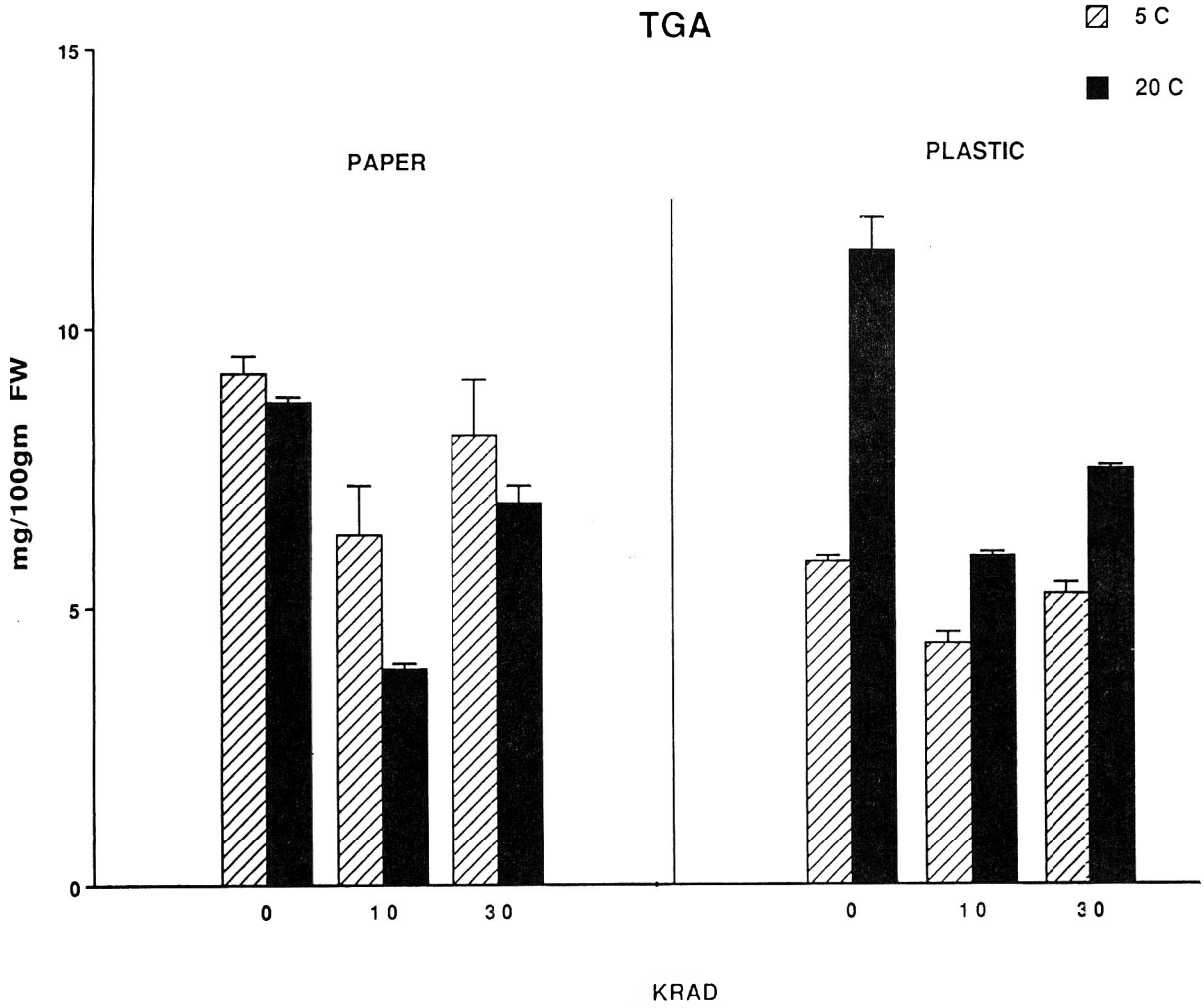

Fig. 2—Effect of temperature on total glycoalkaloids (TGA) of Russet Burbank potatoes stored in different packaging materials for one month. Least significant difference within treatments at $p < 0.005$. Means of four determinations.

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A Research Note

Six Northwest Atlantic Finfish Species as a Potential Fish Oil Source

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ABSTRACT

Lipid, cholesterol, and fatty acid data were determined for six species of fish caught off the coast of Massachusetts on several sampling dates. The fattier fish (>5% fat) had low calculated levels of cholesterol per gram of fat (2–14 mg/g). However, low levels of cholesterol in edible flesh (50 mg/100g) amounted to large amounts of cholesterol per gram fat (17–70 mg/g) for lean fish (<5% fat). Wide fluctuations in fat content of the fatty species were paralleled by changes in fatty acid composition. All species would yield about 35% 20-carbon polyunsaturated fatty acids (PUFA) if the 20-carbon monoenes were removed.

INTRODUCTION

THERE HAS BEEN current widespread interest in therapeutic benefits of fish oils, particularly of omega-3 (ω3) fatty acids provided in fish oils (Kinsella, 1987; Lands, 1986; Nettleton, 1985, 1987). This interest has prompted the search for species which would yield fish oil concentrates highly enriched in polyunsaturated fatty acids (PUFA) when complexed with urea as has been done with menhaden (Ackman et al., 1988). Compositional data on many species have been provided by Sidwell (1981), Gooch et al. (1987), Krzynowek and Murphy (1987), Krzynowek et al. (1989), and Exler (1987). However, extreme fluctuations in observed values (Ackman, 1982) prompted our attempt to reduce geographical variability by sampling at one location on several different sampling dates for six species of finfish. The species chosen are not valued highly for consumption as fillets but are caught in abundance as by-catch. The harvested fish were analyzed for lipid and cholesterol, as well as fatty acid composition to determine their potential as a source of fish oil.

MATERIALS & METHODS

THE SIX FISH SPECIES selected for the study were Atlantic mackerel *(Scomber scombrus),* bluefish *(Pomatomus saltatrix),* sand lance *(Ammodytes americanus),* smooth dogfish *(Mustelus canis),* and spiny dogfish *(Squalus acanthias).* Menhaden *(Brevoortia tyrannus)* was included as a standard to compare fatty acid data (Ackman et al., 1988). Catch data and sample preparation have been described previously (Krzynowek et al., 1990). Although seasonal sampling was intended, very little was done because of the seasonality of the fishery with the exception of bluefish and perhaps spiny dogfish. Identical sampling dates, such as 6/86 for bluefish, reflect two different sampling days within the same month. Lipid content and fatty acid composition were analyzed in duplicate. Cholesterol was extracted from fish tissue in triplicate by the method of Kovacs et al. (1979). Lipids were extracted by modifying the final solvent ratio of the Bligh and Dyer (1959) method to 2:1:2. A portion of the chloroform layer was evaporated to dryness and weighed to determine fat content. Fatty acid composition and cholesterol content were determined by methods described by Krzynowek and Panunzio (1989).

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RESULTS & DISCUSSION

FAT CONTENT (Table 1) is an important consideration in the economics of fish oil production. Smooth dogfish, at <1% fat, probably would not be considered an economical choice due to low yield. As expected, fat content for the fatty fish was variable within a given species. Mackerel and bluefish, for example, ranged from "lean" to "fatty" fish in classification (Stansby, 1982).

Also of interest in the production of fish oils was the amount of cholesterol and the fatty acid composition. Cholesterol, in a fish oil product intended for sale for its therapeutic value in prevention of cardiovascular disease, is viewed as a component that must be eliminated at a certain cost. The amount of cholesterol calculated to be in the oil from the lean fish (<5% fat) was considerably higher (range 17–70 mg/g) than in the oil from the fatty (>5% fat) fish (2–14 mg/g). The cholesterol values at the high end of the latter range were found in menhaden and sand lance, the only two species analyzed as whole fish, and reflect inclusion of bone marrow and gut contents in the total tissue analyzed (Krzynowek et al., 1984).

Fatty acid composition is reported in Table 2. Those fatty acids purported to be of therapeutic value, the ω3 fatty acids, are primarily associated with phospholipids. Therefore, the leaner fish, with a larger proportion of fatty acids as phospholipids, would have a larger proportion of fatty acids present as the ω3 fatty acids. In the case of smooth dogfish, for example, the tissue was about 0.7% fat, primarily phospholipids. The ω3's in smooth dogfish represented about 50% of the total fatty acids. Mackerel was at the other end of the spectrum, with 17% fat and 20% of the total fatty acids present as ω3 fatty acids. However, for every 100g of tissue, mackerel would supply more ω3's than smooth dogfish, because mackerel contained so much more fat. A detailed formula for calculation of fatty acid content in tissue was outlined by Exler et al. (1975) and Weirauch et al. (1977).

Table 1—Lipid and cholesterol content for six species of finfish captured at different times in Massachusetts coastal waters[a]

Species[b]	Date	Fat %	Cholesterol mg/100g tissue	Cholesterol mg/g fat
Mackerel	6/86	3.6 ± 0.3	61.9 ± 1.4	17.2
	8/86	17.3 ± 0.1	36.5 ± 1.6	2.1
	9/86	13.7 ± 1.0	44.8 ± 1.2	3.3
Bluefish	9/85	2.5 ± 0.0	55.2 ± 1.6	22.1
	1/86	17.8 ± 0.5	43.3 ± 1.7	2.4
	6/86	3.6 ± 0.2	63.2 ± 1.1	17.6
	6/86	6.1 ± 0.3	48.8 ± 3.9	8.0
Sand lance	7/86	11.9 ± 0.1	127.8 ± 7.0	11.7
	8/86	9.6 ± 0.6	133.4 ± 4.5	13.9
	8/86	9.5 ± 0.1	138.0 ± 2.0	14.5
Menhaden	8/86	15.8 ± 0.6	91.0 ± 1.0	5.8
	9/86	10.9 ± 0.6	100.3 ± 3.3	9.2
Smooth	9/85	0.7 ± 0.0	48.4 ± 2.3	69.1
Dogfish	10/85	0.7 ± 0.0	33.8 ± 0.6	48.3
Spiny	10/85	12.4 ± 0.4	n.d.	
Dogfish	6/86	17.9 ± 0.7	51.3 ± 2.8	2.9
	8/86	9.0 ± 0.6	40.9 ± 2.6	4.5
	8/86	9.4 ± 0.2	37.4 ± 4.3	4.0

[a] Mean ± one standard deviation.

[b] Menhaden and sand lance sampled whole. All others skinned and filleted.

Table 2—Weight percent of total fatty acid methyl esters (FAME) identified in lipids of finfish caught in Massachusetts coastal waters.

Species[a]	Date	14:0	16:0	16:1 ω7+5	18:0	18:1 ω9+7	18:3 ω3	18:4 ω3	20:1 ω11,9,7	20:4 ω6	20:5 ω3	22:1 ω13,11,9	22:5 ω3	22:6 ω3
Mackerel	6/86	5.3	14.8	4.6	3.4	12.2	0.8	2.4	9.1	0.6	8.4	14.0	1.5	15.1
	8/86	6.6	10.3	8.2	1.2	7.1	0.9	2.6	16.7	0.2	6.6	23.0	1.0	8.2
	9/86	7.4	11.9	4.4	1.6	7.8	1.4	4.1	12.8	0.4	6.0	22.2	1.0	9.8
Bluefish	9/85	3.6	18.8	3.4	6.6	20.8	0.4	0.6	5.3	1.0	3.4	8.3	1.7	16.7
	1/86	0.7	16.1	7.3	3.6	17.2	1.3	2.0	6.7	0.7	7.2	8.7	2.4	10.5
	6/86	5.6	18.2	5.4	4.0	15.8	0.6	1.8	6.4	1.0	6.7	7.8	2.0	17.7
	6/86	7.6	13.8	5.0	3.0	13.0	1.0	2.7	9.4	0.6	5.4	12.6	1.6	13.6
Sand	7/86	8.4	14.2	5.2	1.4	7.8	2.4	8.1	6.2	0.4	10.1	11.2	0.6	13.4
lance	8/86	9.0	13.4	5.4	1.4	6.8	2.5	7.6	6.8	0.5	9.4	12.3	0.6	12.8
	8/86	9.5	12.9	5.6	1.2	6.5	2.7	7.6	6.4	0.6	10.1	10.9	0.7	13.5
Menhaden	8/86	8.0	16.0	9.3	2.8	14.3	0.6	2.2	2.1	0.9	17.8	1.0	2.8	8.2
	9/86	7.2	16.2	8.6	3.1	12.8	0.9	2.9	2.2	0.7	17.2	0.8	2.8	10.9
Smooth	9/85	0.3	15.0	1.8	8.6	13.2	neg	neg	0.8	2.6	6.4	neg	8.0	23.4
dogfish	10/85	0.2	19.0	1.2	10.6	10.5	neg	neg	0.6	4.6	6.0	neg	6.6	25.8
Spiny	10/85	1.1	15.2	3.6	2.6	19.2	0.6	0.8	9.4	2.7	6.8	7.2	2.7	16.0
Dogfish	6/86	1.5	14.3	4.8	2.0	18.4	0.8	0.7	12.4	2.1	7.7	8.8	2.3	16.2
	8/86	1.4	16.0	4.8	2.1	19.6	0.6	0.9	9.5	2.3	8.3	6.0	3.0	15.9
	8/86	2.6	14.8	4.6	2.3	14.8	1.0	2.0	11.4	2.3	8.6	9.1	1.4	15.2

[a] Menhaden and sand lance sampled whole. All others skinned and filleted.

Also, in fatty fish species, as amount of fat increased, exogenous fatty acids began to appear. For example, the relatively high amount of 18:4ω3 (8%) in sand lance was attributed to the food used by these primary herbivores (Ackman and Eaton, 1971). Also, as fat content increased in the flesh of mackerel, the abundance of the monoenes, 20:1 and 22:1, increased, in part contributed by copepods, the main food supply of mackerel. Conversely, since the amount of phospholipids remained fairly constant, the mackerel sample at 3.6% fat showed the larger contribution of the phospholipids to fatty acid composition with its elevated amounts of 22:6ω3.

Smooth dogfish had an unusually high amount of 22:5ω3, an intermediate fatty acid in the retroconversion of 22:6ω3 to 20:5ω3. The large amounts of 22:5ω3 and 22:6ω3 and the virtual absence of the shorter chain ω3 fatty acids was indicative of longer-lived species. Smooth dogfish also had unusually elevated amounts (10%) of 18:0. This acid was generally about 4% of the total.

Menhaden oil had very little (about 3%) of the 20:1 and 22:1 monoenes. Because these monoenes were primarily exogenous, Ackman et al. (1988) suggested that menhaden oil fatty acid composition, with its negligible amounts of 20:1 and 22:1 fatty acids, most closely represented a "basic" fish oil composition that could be achieved with other oils through elimination of monoenes with urea fractionation. If these monoenes were subtracted from the total contribution of all the fatty acids for the fish in our study, all but the smooth dogfish, would resemble menhaden oil. Smooth dogfish had about 50% of 20-carbon PUFA. The menhaden samples included in our study resembled those of Dubrow et al. (1976) and Ackman et al. (1988) with the ω3 fatty acids representing about 30–35% of the total fatty acids.

All species, with exception of smooth dogfish, could be considered in Stansby's (1982) category B (medium oil, 5–15%) and occasionally in category C (high oil, >15%) and were either good candidates for or already in use for fish oil production. Their fatty acid composition, after subtraction of the monoenes, closely resembled that of menhaden with ω3 accounting for about 35% of total fatty acids. Smooth dogfish with low fat (0.7%) and high cholesterol levels (about 60 mg/g oil) probably would not lend iteself for this use in spite of the high proportion of ω3 fatty acids (about 50%).

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A Research Note

Gamma Irradiation Effects on Shelf Life and Gel Forming Properties of Washed Red Hake (*Urophycis chuss*) Fish Mince

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ABSTRACT

Fresh washed red hake (*Urophycis chuss*) mince without cryoprotectants was irradiated at 0 (control), 0.66 and 1.31 kGy and stored aerobically at 3.3°C. The total aerobic plate counts of the control and the low and high levels irradiated samples remained less than 10^6 CFU/g for 4, 10, and 17 days, respectively. Gel strength decreased after irradiation of mince, and such decreases were dose-dependent. Irradiation extended sensory shelf life of unfrozen fish mince 12–18 days and microbiologically ($< 10^6$ CFU/g) 6–13 days longer than the unirradiated control.

INTRODUCTION

FROZEN FISH MINCE SURIMI is used as an intermediate raw material for manufacture of a variety of fabricated seafood products (Lee, 1984). Currently, fish mince can be cryostabilized only with cryoprotectants such as sucrose and/or sorbitol during frozen storage without losing the functional properties necessary for gel formation. As the surimi industry continues to grow, interest has risen in developing fresh mince-based products such as fishburger with no added sweetening stabilizers.

However, fresh fish mince is perishable and requires treatment for adequate shelf-life needed for distribution and storage before processing. Irradiation appeared to be the most promising method which could extend shelf life without freezing or significant changes in functional properties. Although the FDA approvals for food irradiation did not at present include fish, the prospect of such approval was good based on scientific data on safety of irradiated seafoods.

Summaries of numerous studies indicating the wholesomeness and applications of irradiated seafoods have been published by the IFT Expert Panel on Food Safety and Nutrition (1983), the Council for Agricultural Science and Technology (CAST, 1986, 1989), the World Health Organization (WHO, 1981, 1988), the IFT Symposium (1989), Newsome (1987), and Diehl (1982, 1990). Seafood irradiation research was extensively reviewed (Nickerson et al., 1983). Studies relating to the effect of irradiation on fresh fish mince preservation and gel formation were, however, lacking. The only related studies were published by Sasayama (1972) who reported an increase in storage life of irradiated fried kamaboko from frozen ground sardines.

Our study was designed to determine: (1) the appropriate level of irradiation for pasteurization of fresh fish mince, and (2) the effect of dose level on microbial growth, sensory acceptance, gel-forming properties, and overall shelf-life extension of fresh fish mince without added cryoprotectants.

MATERIALS & METHODS

WASHED FISH MINCE was prepared from fresh red hake (*Urophycis chuss*) (less than 2 days old), following the procedure used by Lee (1986). The refined mince was vacuum-packed in nylon bags and stored on ice 12 hr before irradiation.

Irradiation was carried out on two separate occasions at the University of Lowell Radiation Laboratory Cobalt-60 gamma facility (Lowell, MA). Mince was irradiated at 0, 0.66 and 1.31 kGy. The dosimeters used were Optichromic dosimeters which the University of Lowell calibrated and were traceable to the National Institute of Standards and Technology of the U.S. Department of Commerce. Temperature rise in irradiated samples was recorded with a digital thermometer immediately after irradiation. All samples were cooled to 3.3°C and maintained there throughout the study. After opening the bag for sampling, anaerobic conditions were no longer maintained.

Microbiological evaluations of control and irradiated samples were performed on 0, 4, 10, 17, and 24 days after irradiation. Total aerobic plate count (APC) was obtained from duplicates of plate count agar. Total anaerobic population was obtained from counts on thioglycollate-agar plates incubated in anaerobic culture jars.

For texture analysis, the mince was made into surimi-type gels at 3-day intervals until 24 days, unless it became sensorially objectionable sooner. The gels were formed by adding 2% salt to the mince, comminuting, extruding into casings, and steam-cooking (90°C) for 20 min. The formed cylindrical gels (25 × 25 mm) were tested on an Instron testing machine (model 1122) for measurement of compressive force at failure (90% deformation) and penetration force using methods described by Lee and Chung (1989). Radiation and tests for compressive and penetration forces were replicated twice.

Sensory evaluation of the mince was performed in four sessions by an expert panel of five familiar with surimi. Samples were prepared by placing 25g mince in aluminum dishes and steaming 5 min. Panelists were asked to rate the samples as "acceptable" or "not acceptable."

RESULTS & DISCUSSION

Microbiology

Selection of radiation doses (0.66 kGy and 1.3 kGy average doses) was based on a recommendation to FDA by Ronsivalli and Kaylor (1981) to restrict radurization of chilled fresh seafood to the 0.75 to 1.5 kGy dose range. They reasoned in that dose range sufficient numbers of spoilage organisms were killed to achieve a 7–10 days extension in refrigerated shelf life at 0 to 3°C. At the same time, sufficient numbers would survive to assure that the product would spoil 5 to 7 days before any possible *Clostridium botulinum* Type E spores could grow out to make it toxic.

Irradiation immediately reduced the total aerobic plate counts (APC) from 10^5 CFU/g (day 0, unirradiated control) to 10^3 CFU/g for both mince samples irradiated at 0,66 kGy and 1.3 kGy (Fig 1). While stored at 3.3°C, the unirradiated control had a rapid build-up of aerobic microorganisms from 10^5 CFU/g at day 0 to almost 10^6 CFU/g at day 4 and 10^7 CFU/g at day 10, when it was discarded as spoiled with a strong offensive odor.

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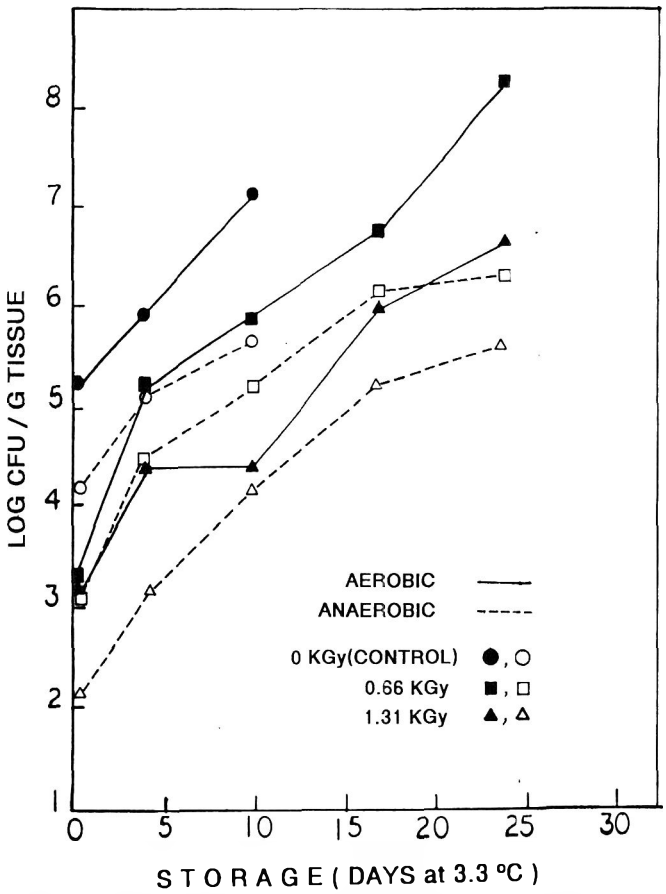

Fig. 1—Total aerobic and anaerobic microbial populations of control and irradiated fish mince during storage at 3.3°C.

Aerobic microorganisms in mince irradiated at 0.66 kGy increased to almost 10^6 CFU/g in 10 days, and continued to increase with the 24-day termination, when the count reached 10^8 CFU/g (Fig. 1). This high count could have been due to proliferation of relatively irradiation resistant microorganisms after the more irradiation sensitive members were killed. Another possibility was the recovery and growth of some sublethally injured microorganisms.

As shown in the same figure, facultatively anaerobic plate counts followed the same pattern as the aerobic plate counts except they were lower. Reductions in aerobic and anaerobic microorganisms were in line with conclusions of Nickerson et al. (1983) that irradiation dose levels between 1.0 and 2.5 kGy were generally effective in reducing microorganisms and extending shelf life in marine fish species. While radurization (pasteurization) will kill spoilage bacteria, it is important to emphasize that this low-level treatment would not destroy spores or toxin of type E *Clostridium botulinum* (Eklund, 1982).

Microbiological and sensory shelf life

Because of constraints in using volunteers as test subjects at the University of Rhode Island and for esthetic reasons, the sensory evaluation panel established objective "unacceptable quality" cut-offs when APC exceeded 10^6 CFU/g for unirradiated or irradiated mince, in addition to subjective sensory factors of odor and taste. In the unirradiated control, the 10^6 CFU/g APC coincided with formation of objectionable spoiled fish odor after 6 days storage at 3.3°C.

On the basis of APC of 10^6 CFU/g as cut-off and sensory panel ratings, the shelf-life of 0.66 kGy irradiated samples was estimated to be 10 and 18 days, respectively, an extension of 6 and 12 days over the unirradiated control (4 and 6 days). As the sample irradiated at 1.31 kGy had minimal sensory changes and an APC of nearly 10^6, shelf-life was estimated to be at least 17 and 24 days, respectively, an extension of 13 and 18 days.

Our findings that substerilizing doses of 0.66 and 1.31 kGy reduced concentrations of microorganisms and extended shelf life of fish mince for 6 to 13 days substantiated a number of previous studies (Spinelli et al., 1965; Ronsivalli et al., 1969; Venugopal, et al., 1987).

At the levels of ionizing energy used, no irradiation-induced odor or flavor was noted by the expert sensory panel. We were not surprised that the expert taste panel members could not easily detect possible incipient spoilage in the irradiated mince. In spite of an APC of 10^6 CFU/g, the lack of offensive odor and flavor changes suggested that the quality cut-off point for irradiated fish may be an APC as high as 10^7 CFU/g. This observation has also been reported by Spinelli et al. (1964, 1965), Corlett (1967), and Laycock and Regier (1970). However, these observations may indicate a need for a safety margin in microbiological standards for irradiated fishery products.

Analysis of gel-forming ability

As shown in Table 1, irradiation of the mince caused immediate decreases in compressive force by 28 and 48.8%, and to a lesser extent, penetration force values by 17.3 and 21.1% at 0.66 and 1.31 KGy, respectively. After 3 days storage at 3.3°C, compressive and penetration forces were further reduced for the control and irradiated mince. We believed this was due to proteolytic degradation of gel-forming myofibrillar proteins. Thereafter, from storage day 3 to day 15, the gel strength values remained unchanged. These results clearly indicated that irradiation, depending upon dose level, decreased the ability of mince to form highly elastic gels. This finding was supported by a previous report that irradiation caused meats to become softer and more tender (Bailey and Rhodes, 1964). Texture softening after irradiation was ascribed to degradation of proteins by such reactions as deamination and scission of peptide and disulfide bonds.

CONCLUSIONS

IRRADIATION of fresh fish mince with 0.66 and 1.31 kGy extended shelf life 6 and 13 days, respectively. Such extension would allow more time between mince processing and manufacture of finished products without freezing. Because of the destruction of those microorganisms which cause objectionable odors and flavors, a taste panel rated irradiated mince having rather high total aerobic plate counts as sensorially "acceptable." This observation may indicate the desirability of having

—Continued on page 1748

Table 1—Gel-forming properties of irradiated red hake mince during storage at 3.3°C

Irradiation dose (kGy)	Storage (days)					
	0	3	6	9	12	15
	Compressive/penetration force (kg)					
0	25/0.52	12/0.35	—	—	—	—
0.66	18/0.43*	7.5/0.29*	9.5/0.27	8.5/0.30	6.7/0.31	6.5/0.29
1.31	12.8/0.41*	5.5/0.28*	3.5/0.26	6.0/0.30	4.5/0.26	7.5/0.28

* Significantly different from the controls ($p<0.01$, $n=4$)

A Research Note

Initial Fish State and Mixing Time Effects on Textural Characteristics of a Restructured Catfish Product

G.P. ABIDE, J.O. HEARNSBERGER, and J.L. SILVA

ABSTRACT

A restructured catfish product similar to a corn dog was developed using tumbled catfish from low cost fillets. The product when made from fresh fillets, was more tender but had tougher skin than when made from frozen fillets. Mixing more than 5 min resulted in a more rubbery and tougher product as demonstrated by higher Instron peak load and longer relaxation time.

INTRODUCTION

RESTRUCTURED muscle products have been developed using red meats, poultry and seafood. In general, production of such products has involved some type of mixing of size-reduced meat ingredients to aid in extraction of proteins which serve as a binding agent (Addis and Schanus, 1979). Time of mixing has affected the textural properties of the finished product, with longer mixing times resulting in increased adhesion between meat pieces (Booren et al., 1981).

Many methods have been used to objectively measure binding strength in restructured muscle foods. A tensile test has been used by many (Macfarlane et al., 1977, Weinberg, 1983). Recently, others have proposed use of a stress-relaxation test (Weinberg and Angel, 1985; Hamman, 1988). The purpose of this study was to use a stress-relaxation test to compare viscoelastic properties of a restructured catfish product made with fresh or frozen catfish using different mixing times.

MATERIALS & METHODS

FRESH CHANNEL CATFISH (*Ictalurus punctatus*) was obtained from the processing line and held at 2°C for 6 hr; frozen catfish was obtained after being individually quick frozen and held at −20°C for 24 hr. The fish were obtained on the same day from a commercial Mississippi catfish processor and transported in ice (fresh) or dry ice (CO_2) (frozen) to the MSU Food Processing Laboratory for preparation and analysis. Fish from each lot were cut into cubes approximately 2.5 cm × 1.2 cm. The fish were weighed into 5 kg batches, and 50g NaCl, 25g ground black pepper and 12g natural lemon flavor (MCP Foods, Anaheim, CA) were added to each. Batches were then mixed for either 1, 5, or 9 min in a Hobart Model A-200 food mixer using a dough hook attachment (Hobart Corp., Troy, OH). The mix was stuffed into 32 mm clear cellulose casings using a piston driven sausage stuffer, linked into 7.6 cm portions, and soaked in 1% acetic acid solution for 1 min, to aid in casing removal after cooking. After soaking, the stuffed fish was rinsed in cool water, hung in a gas heated smokehouse, and cooked in a dry atmosphere at 93°C to 66°C internal temperature. After cooking, the links were held overnight at 2°C, then casings were removed by hand. The links were then battered using a cornmeal based batter and fried in vegetable oil at 183–188°C for 6–7 min.

After cooling, the batter was removed and a 2×2×3cm sample taken from the center of each link. An Instron Universal Testing Machine Model 1011 (Instron Corp., Canton, MA) with a 5 kg load transducer equipped with a plunger was used to conduct a stress-relaxation test on each sample. In this test, the sample was placed on a solid surface with the tallest side in a vertical position. The crosshead and chart speed were 20 cm/min, and the level of deformation was 50% (1.5 cm) of the original height. The force required to achieve 50% compression was reported as peak load. The relaxation time was defined as the time required for the peak stress force to decrease by 50%.

Five trained panelists rated skin toughness, and chewiness of fish meat using an unstructured 15-point (15 cm long) scale (Stone et al., 1974). Higher scores indicated softer skin, more rubbery, and softer product, respectively.

This was a split plot in a completely random design with whole plot treatments being the initial fish state (fresh vs frozen). Sub-plot treatments consisted of the 3 mixing times (1, 5, or 9 min). The experiment was replicated 3 times. Analysis of data was conducted using SAS (1985) GLM procedures.

RESULTS & DISCUSSION

NO DIFFERENCES were found in stress force peak values or relaxation times between product made with fresh or frozen fillets (data not shown). Peak load (Table 1) increased ($P<0.05$) with increased mixing time, indicating progressive hardening of the product. This was the result of greater extraction of salt-soluble proteins with mixing time, thus more binding of product. The relaxation time (Table 1) increased ($P<0.05$) for the product mixed 9 min, indicating a more elastic product as compared to the 1 and 5 min mixed products. This could be attributed to the greater amount of salt-soluble proteins extracted.

Taste panelists rated more rubbery ($P<0.05$) the products with increased mixing time (Table 1). These findings agreed with results of increased peak load and relaxation time.

Restructured products made from frozen fish showed a lower ($P<0.05$) peak load than their counterparts made from fresh fish (Table 2). This was confirmed by taste panelists who rated

Table 1—Sensory and textural characteristics of a restructured catfish product as affected by mixing time

Mixing time (min)	Peak load[a] (N)	Relaxation time[a] (sec)	Sensory attributes	
			Chewiness[b]	Skin toughness[c]
1	11.8	14.1	7.9	8.4
5	13.7	15.2	8.0	7.4
9	15.6	19.4	9.3	6.5
LSD(0.05)	1.7	4.3	1.0	1.6

[a] Peak load and relaxation time after 50% deformation.
[b] Scores were 1.2 = soft and 13.8 = rubbery.
[c] Scores were 1.2 = tough and 13.8 = tender.

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the product from frozen fish more rubbery than from fresh fish.

Skin toughness, determined by taste panelists, decreased ($P < 0.05$) after 9 min mixing time (Table 1) and was lower for products made with frozen fish (Table 2). A restructured catfish product could be made from fresh or frozen fish. The product was less rubbery when made from fresh fish and when mixed for the least time. However, longer mixing time resulted in better binding of the product.

Table 2—Sensory and textural characteristics of a restructured catfish product as affected by initial fish state

Initial fish state	Peak load[a] (N)	Sensory scores	
		Chewiness[b]	Skin toughness[c]
Fresh	2.45	7.4	8.7
Frozen	3.24	9.3	6.1
LSD (0.05)	0.39	1.6	1.9

[a] Peak load after 50% deformation.
[b] Scores were 1.2 = soft and 13.8 = rubbery.
[c] Scores were 1.2 = tough and 13.8 = tender.

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a safety margin in microbiological standards for irradiated fishery products.

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A Research Note

Application of Ellipsometry to Evaluate Surface Cleaning Effectiveness

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ABSTRACT

Mechanisms associated with the cleaning of food contact surfaces are not completely understood, and attempts to evaluate or improve cleaning processes are consequently hindered. The usefulness of ellipsometry as a technique to rate efficiency of different cleaning methods with respect to ability to remove milk soils from various materials is described. Film properties obtainable with ellipsometry, including a measure of the apparent mass remaining on a given surface after cleaning, can be used to provide an indication of the relative effectiveness of a cleaning method. This information contributes to establishment of criteria by which the effectiveness of a cleaning operation can be judged.

INTRODUCTION

CLEANING OF FOOD contact surfaces is a nearly continuous activity associated with a pronounced economic impact. Nevertheless, our understanding of the mechanisms involved is still incomplete, and attempts to improve cleaning process are consequently hindered. Many studies have contributed to a better understanding of some fundamental aspects of cleaning. Reports related to contact material surface chemistry, transport properties important to cleaning, and cleaning kinetics have been published in the proceedings of two international conferences (Hallström et al., 1981; Lund et al., 1985). The third in this series of international conferences on fouling and cleaning in food processing was held in 1989 in Munich.

Techniques based on ellipsometry have been used to carry out investigations in the general area of hard surface cleaning (Engström and Bäckström, 1987; Bäckström et al., 1988). Ellipsometry is an optical technique used to determine the thickness and refractive index of thin films; it enables the continuous monitoring of adsorption and desorption processes at interfaces (Archer, 1968). Basically, a laser beam of known physical properties is transmitted to a film-covered surface and reflected. Physical properties of the beam change upon reflection, and these changes are measured. The measured differences between properties of the incident and reflected beams are totally dependent on sample film thickness and refractive index; these film properties are evaluated simultaneously with the aid of a microcomputer interfaced to the ellipsometer. Knowing film thickness and refractive index, adsorbed mass can be calculated by the Lorentz-Lorenz relationship as experimentally verified by Cuypers et al. (1983). Optics associated with ellipsometric instrumentation, ellipsometric theory and descriptions of numerous applications have been published elsewhere (McCrackin et al., 1963; Azzam and Bashara, 1977). In this report, we describe the usefulness of ellipsometry for characterizing milk soils on fouled surfaces as well as those remaining on "cleaned" surfaces. Application of the technique to rate efficiency of different cleaning methods with respect to their ability to remove milk soils from various materials is detailed.

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MATERIALS & METHODS

FIVE SAMPLE PLATES (approximately 1 cm × 2 cm) were constructed from each of five different materials: #304 stainless steel, glass, polycarbonate, polyester, and acrylic. The surfaces of the polymers were protected during shipment from the supplier and during storage with a protective polymeric film applied by the manufacturer. The #304 stainless steel was polished to a mirror finish. All sample plates were immersed in acid dichromate cleaning solution with ultrasonic treatment for 10 min, rinsed with deionized water and dried at room temperature in a desiccator prior to use.

The polymers as well as the glass were transparent. During ellipsometric analysis, to prevent reflection of the laser beam from the "back" of these materials (i.e., the surface opposite that being analyzed), one side of each was blackened evenly with spray paint. Substrate constants (refractive indices of the bare surfaces required for ellipsometric evaluation of film thickness and refractive index) of each plate were then determined with ellipsometry.

Fouling and cleaning procedures

The five sample plates of each material were independently immersed in raw, whole milk (Miller's Dairy, Harrisburg, OR) at 40°C for 30 min with agitation. The materials were subsequently rinsed in tap water for 10 sec at room temperature, and dried overnight in a desiccator.

The thickness and refractive index were ellipsometrically determined for the films formed on each material. In each case, multiple readings were made at each of about ten different surface locations. These data were used to calculate the average value of mass adsorbed on each surface.

The five sample plates representative of each material were then individually cleaned by one of five different methods: *water*—samples were immersed in tap water at 40°C for 10 min with agitation; *phosphoric acid*—samples were immersed in a 25% H_3PO_4 (J.T. Baker Chemical Co., Phillipsburg, NJ) solution at 40°C for 10 min with agitation; *MCDS & surfactant*—samples were immersed in an aqueous solution of monocarbamide dihydrogen sulfate and a proprietary surfactant (Unocal Chemicals Division, Los Angeles, CA) prepared such that the concentration of H_2SO_4 was 25% and that of surfactant was 5% by mass; contact was for 10 min at 40°C with agitation; *alkaline-acid detergent*—a two-step process in which samples contacted a chlorinated alkaline cleaner (Cold-War®, Klenzade Division, Economics Laboratory, Inc., St. Paul, MN) for 5 min at 40°C with agitation followed by a 10-sec tap-water rinse and contact with an acid detergent (PL-3, Klenzade Division, Economics Laboratory, Inc., St. Paul, MN) for 5 min at 40°C with agitation; and *alkaline-MCDS*—a two-step process similar to that used above with the exception that the phosphoric acid detergent was replaced by MCDS (in the absence of surfactant) at an equivalent acidity.

The products were used as recommended by the manufacturer. The alkaline cleaner was prepared at the rate of 1 ounce : 3 gallons of tap water (pH = 11.83). The acid detergent was prepared at 1 ounce : 5 gallons of tap water (pH = 2.59). When used in place of acid detergent, MCDS was used at 1 ounce : 20.7 gallons of tap water (pH = 2.59). Each of the single-step cleaning methods was followed by a 20-sec rinse with tap water; the two-step methods were followed by a 10-sec rinse. All materials were subsequently placed into a desiccator and dried overnight. The thickness and refractive index of the films re-

Table 1—Mass of milk soil present on each surface (μg cm^{-2}) after immersion in milk, and following cleaning by methods listed at left[a]

	F_1	C_1	% rem.	F_2	C_2	% rem.	F_3	C_3	% rem.
acrylic									
water	3.80	3.73	1.8	6.93	5.47	21.1	7.03	4.71	33.0
phosphoric acid	7.62	6.45	15.4	4.98	2.86	42.6	4.57	3.73	18.4
MCDS & surfactant	2.85	2.44	14.4	3.89	1.93	50.4	5.18	5.81	–
alkaline-acid detergent	4.96	0.44	91.1	0.60	0.15	75.0	3.98	1.51	62.1
alkaline-MCDS	5.12	1.32	74.2	7.26	1.22	83.2	7.28	3.85	47.1
glass									
water	5.11	5.01	2.0	4.34	2.25	48.2	4.62	4.06	12.1
phosphoric acid	4.60	2.67	42.0	2.77	1.71	38.3	4.43	3.25	26.6
MCDS & surfactant	5.14	4.60	10.5	2.57	2.05	20.2	4.89	1.22	75.1
alkaline-acid detergent	3.59	1.14	68.2	3.30	1.45	56.1	5.03	1.36	73.0
alkaline-MCDS	1.31	1.74	–	2.98	1.40	53.0	4.87	3.22	33.9
polycarbonate									
water	7.40	4.81	35.0	5.66	1.32	76.7	11.8	4.33	63.3
phosphoric acid	10.4	0.57	94.5	5.12	0.78	84.8	11.5	3.12	72.9
MCDS & surfactant	6.04	3.54	41.4	5.31	0.21	96.0	8.72	4.95	43.2
alkaline-acid detergent	7.81	0.25	96.8	5.39	1.28	76.3	8.33	3.09	62.9
alkaline-MCDS	5.00	3.33	33.4	7.45	3.86	48.2	10.5	2.11	79.9
polyester									
water	5.18	4.96	4.2	7.36	8.85	–	7.83	6.20	20.8
phosphoric acid	8.71	5.55	36.3	7.96	7.33	7.9	8.02	7.40	7.7
MCDS & surfactant	9.67	5.92	38.8	9.88	6.41	35.1	4.54	7.50	–
alkaline-acid detergent	6.78	1.66	75.5	8.31	4.90	41.0	7.39	5.55	24.9
alkaline-MCDS	4.22	3.49	17.3	9.88	3.03	69.3	9.69	7.01	27.7
stainless steel									
water	6.23	4.95	20.5	3.06	3.48	–	4.10	3.72	4.4
phosphoric acid	2.93	1.96	33.1	2.05	1.14	44.4	1.91	1.82	4.7
MCDS & surfactant	3.76	0.33	91.2	0.72	0.45	37.5	4.04	2.15	46.8
alkaline-acid detergent	2.15	0.43	80.0	2.21	1.49	32.6	3.38	2.13	37.0
alkaline-MCDS	3.19	0.72	77.4	3.55	1.87	47.3	3.03	0.80	73.6

[a] Columns headed by F_1 and C_1 refer to the mass of milk soil present on each surface after the ith fouling (F) or cleaning (C) step. For each surface, the fraction of soil removed by each cleaning method, expressed as % rem., is also tabulated.

maining on each material were ellipsometrically determined. Again, multiple readings were made at each of about 10 different surface locations on each sample plate, and the data were used to calculate an average value of mass adsorbed on each surface and its associated standard error. The cycle of (1) immersion in milk, (2) analysis of films formed, (3) cleaning, and (4) analysis of films remaining, was performed three times with the same surfaces.

RESULTS & DISCUSSION

Cleaning effectiveness

The results are presented in Table 1. The columns headed by F_i and C_i refer to the mass of milk soil present on each surface after the ith fouling (F) or cleaning (C) step. For each surface, the fraction of soil removed by each cleaning method, expressed as % removed, is also tabulated. Two criteria must be met in exact ellipsometry: (1) the surface that supports the film must be specular and (2) the films must be homogeneous. All surfaces used in this work were sufficiently specular, but milk films are never homogeneous. Consequently, calculated values of adsorbed mass shown in Table 1 are relative, and specific to each type of material. This can be explained with reference to the Lorentz-Lorenz relationship. The value used for the required molecular weight/molar refractivity ratio for β-lactoglobulin was 4.10 g cm^{-2}, equivalent to that calculated by Cuypers et al. (1983). The tabulated values are in fact representative of an apparent mass of β-lactoglobulin adsorbed. Film chemical composition is expected to be similar on identical surfaces and varies from one type of surface to another; the recorded data are therefore material-specific. For all surface films considered, the standard errors associated with the estimates of adsorbed mass were completely normal, i.e., at least one order of magnitude less than the value of adsorbed mass. They were not shown in Table 1 because of space limitations.

Thickness and refractive index values were evaluated at ten or more regions on each surface film analyzed. Each thickness and refractive index pair enabled calculation of the adsorbed mass in that region. Note that each thickness and refractive index pair used to compute adsorbed mass represented an average of several readings on that region of the surface; consequently, the associated value of adsorbed mass was itself a mean value. The values reported in Table 1 are the averages of these region-specific mean values of adsorbed mass.

Although contact between milk and samples of any of the five virgin materials resulted in varying amounts of film formation (column F_1) such scatter was expected due to the non-isotropic nature of the films and surfaces. With few exceptions, horizontally from the initial value of surface coverage to the last column of Table 1, surface coverage decreased with cleaning and increased upon reimmersion in milk, as intuitively expected. Moreover, in most cases the amount of soil remaining on each surface after cleaning increased as the cycle progressed. This observation was not well established with these data, however, most probably due to the fact that the films were generated under very mild conditions.

The purpose of this paper was not to rate the five cleaning methods; nevertheless, the effectiveness demonstrated by each cleaning method could be quantified according to the average fraction of soil removed from each surface. Based on this criterion, the "overall" most effective cleaning method was the alkaline - acid detergent method, followed by the alkaline - MCDS method. Differences between cleaning properties of MCDS and surfactant, and phosphoric acid were difficult to discern. The water rinse was the "overall" least effective method. Although ellipsometry was not designed for analysis of films characterized by the high degree of heterogeneity displayed by milk soils, for a given contact material a measure of the apparent mass remaining on its surface can be used to provide an indication of the relative cleaning effectiveness among several considered methods.

Character of the films

In addition to apparent adsorbed mass, the more fundamental properties of average film thickness and refractive index can provide information more closely related to cleaning mechanism. General observations related to the thickness and refractive index of each of the milk films analyzed can be adequately described with some specific examples supported by Table 2. As shown in Table 2, the first films formed on each material were of similar average refractive index. More-

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A Research Note

Confocal Scanning Optical Microscopy of Meat Products

P.D. VELINOV, R.G. CASSENS, M.L. GREASER, and J.D. FRITZ

ABSTRACT

Confocal scanning optical microscopy (CSOM) was used to observe the internal structure of frankfurters and summer sausage. This new method allows optical sectioning of thick specimens and thus avoids the potential problem of smearing encountered when fat globules and bacteria are at the surface of sections.

INTRODUCTION

SINCE the early morphological description of meat emulsions by Hansen (1960), numerous attempts have been made to refine histological techniques, quantitate results and relate morphological findings to properties of meat products. With ordinary light microscopy lipid droplets may be damaged and spread across the surface during sectioning, and thus make it difficult to distinguish them from lipid droplets truly embedded in the protein matrix near the cut surface. Similarly, it has been difficult to determine if bacteria are on the surface or actually located within the section. Such possible artifacts have caused problems in interpretation, especially in terms of relating location to structure. The newly developed technique of confocal scanning optical microscopy (CSOM), should alleviate these problems.

The theory of this new technique is reviewed in detail by Shotton (1989), and the ability of the instrument to do optical sectioning has been explained by Wilson (1989). In biological specimens, the optical sections can be obtained noninvasively and are essentially free from out-of-focus blur; the data collected can be processed into a three-dimensional image (Shotton and White, 1989). Heertje et al. (1987) used the technique to observe fat spreads, mayonnaise, cheese and rising dough, and they concluded it was a useful technique to study food microstructure.

We are reporting the use of CSOM to observe structures within thick sections of processed meat.

MATERIALS & METHODS

SAMPLES of commercial frankfurters of about 0.5 cm per side were fixed in 10% formalin for 24 hr. Samples were frozen in isopentane cooled with liquid nitrogen and sectioned at 40 μm thickness in a cryostat at −20°C. Sections were stained in a 0.01% aqueous solution of Nile blue A for 5 to 10 min. Following staining, the sections were rinsed briefly in water to remove excess stain and mounted under sealed coverslips in 70% glycerol containing 1 mg/ml para-phenylenediamine (Johnson et al., 1982) to limit fading. Slides were stored at −20°C in a light tight box.

Thick sections (50–60 μm) of commercial summer sausage were made as described above and stained for 1 to 3 min in 0.1% (w/v) aqueous Acridine Orange (Yiu, 1985) to visualize bacteria.

Microscopy was conducted with a MRC-500 confocal imaging system (Bio-Rad Microscience). For Nile blue A stained sections, filter system FCII (high sensitivity green excitation at approximate wavelength 515 nm), and for Acridine Orange stained sections, filter system FCI (high sensitivity blue excitation at approximate wavelength 490 nm) were used. Photomicrography was carried out using 35 mm Kodak T-Max 400 professional film.

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RESULTS & DISCUSSION

A TYPICAL RESULT for frankfurters is shown in Fig. 1. The fat globules appear as intense well-defined light areas against a dark background. The four views were taken at 0, 5, 10 and 15 μm of depth in the section. A good emulsion is shown with a rather uniform distribution of small fat globules throughout the protein matrix. Figure 2 shows the staining of bacteria, and in this case the optical sections were at 0, 10, 20 and 30 μm. They appear as intensely stained particles. The bacteria, identified by arrows in Fig. 2, had probably taken the characteristic shape because of competition in the fermented product (Leistner and Lucke, 1989).

The point illumination and pinhole detection system effectively suppressed interference from off-focus levels of the objects, and as Heertje et al. (1987) mentioned, this is the main advantage of CSOM compared to conventional light microscopy. Since microscopy was on thick sections, images were observed away from the surface thereby avoiding disruption of internal structure. According to Heertje et al. (1987), CSOM allows visualization to a depth of more than 100 μm under the surface of the thick section so the structures are not deformed. The operation requires only a few minutes.

Because this technique allows viewing below the surface of the section it is especially useful for determining if bacteria exist inside the product or are a contaminant which has been smeared on the surface during sectioning.

Quantitative information about size and spatial distribution of the stained structures can be obtained with appropriate image processing techniques.

Our investigations show CSOM is a promising tool for study of meat microstructure. Optical sectioning affords new oppor-

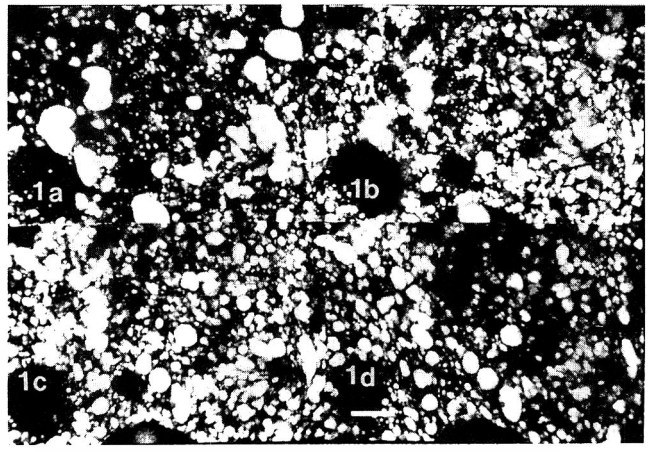

Fig. 1—Internal structure of frankfurter showing size and distribution of fat globules. Optical sectioning is as follows: 1a, 0 μm; 1b, 5 μm; 1c, 10 μm; 1d, 15 μm. Scale bar is 250 μm.

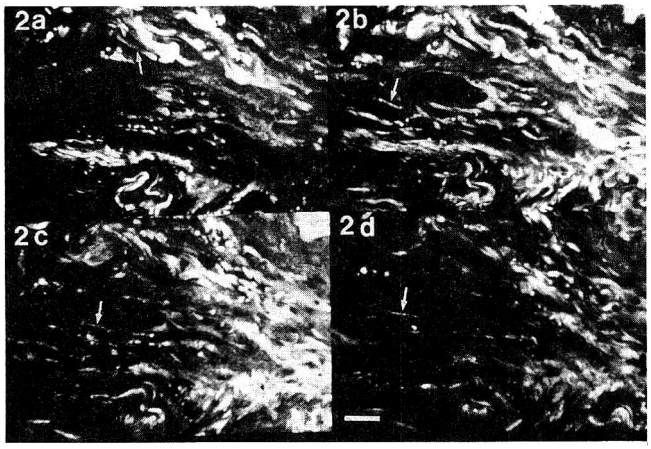

Fig. 2—Internal structure of summer sausage showing bacteria as bright objects. Arrow indicates elongated bacteria. Optical sectioning is as follows: 2a, 0 μm; 2b, 10 μm; 2c, 20 μm; 2d, 30 μm.

tunities for quality control and monitoring the effects of ingredients, equipment and schedules on product characteristics.

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Table 2—Average refractive index, n_f, and film thickness, d (Å), on each material following the initial fouling step and after cleaning with phosphoric acid.[a]

	Fouled		Cleaned	
	n_f	d	n_f	d
acrylic	1.449 (0.001)	693 (26.9)	1.448 (0.001)	587 (56.2)
glass	1.471 (0.003)	402 (38.7)	1.459 (0.009)	236 (39.0)
polycarbonate	1.467 (0.004)	908 (114)	1.440 (0.030)	78 (12.1)
polyester	1.477 (0.003)	754 (57.7)	1.476 (0.001)	479 (32.2)
#304 stainless steel	1.459 (0.071)	321 (47.6)	1.543 (0.061)	181 (33.1)
(2nd fouling & cleaning)	2.362 (0.138)	92 (17.0)	2.068 (0.190)	72 (10.8)
(3rd fouling & cleaning)	2.517 (0.120)	75 (6.1)	2.629 (0.169)	75 (10.6)

[a] The average refractive index and film thickness on #304 stainless steel evaluated during the second and third fouling and cleaning cycles with phosphoric acid are also shown. Standard errors in parentheses.

over, after cleaning by any method in the absence of alkaline detergent, film thickness was reduced but its refractive index remained generally unchanged. In subsequent fouling and cleaning cycles, these findings held true only for glass and the three polymers. Milk films formed on stainless steel behaved somewhat differently; this is also indicated in Table 2. Following the initial fouling and cleaning cycle, films bound to stainless steel were of relatively high average refractive index and not as thick as those on other surfaces. This observation indicates a more compact, tightly bound film. Finally, another consistent observation worth noting was that after cleaning by either of the two methods that included contact with alkaline detergent film thickness was reduced. Also, surfaces exhibited regions where those films were totally removed, i.e., regions in which the "film" refractive index essentially equaled that of air ($n_{air} = 1.000$). This final observation suggested that alkaline contact was required for complete cleaning.

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A Research Note

Correlation of Protein Sulfhydryls with the Strength of Heat-Formed Egg White Gels

B. A. MARGOSHES

ABSTRACT

A strong, direct correlation (r = 0.97) was found between egg white protein surface sulfhydryl group (−SH) concentration and the gel strength of its heat-induced gels. An inverse correlation (r = −0.86) was also found between the −SH group concentration of 0.1% SDS-denatured egg white proteins and gel strength. Data indicated that surface −SH groups played an important role in strong heat-induced gel formation in egg whites.

INTRODUCTION

INVESTIGATION of the factors involved in gelation of egg whites is an active area because of the use of egg whites in a wide variety of food products. Gosset et al. (1984) reviewed many factors affecting egg white gelation, which included electrostatic forces, protein concentration, hydrophobic amino acid residue exposure, the formation of disulfide bonds, and albumen composition. Hirose et al. (1986) demonstrated the formation of gels upon addition of thiol reagents to fresh egg white at low temperatures (10–35°C) and concluded from work with purified egg white fractions that a conalbumin-rich fraction was the major gel-forming component. The purpose of our study was to determine whether a correlation existed between the sulfhydryl (−SH) groups in egg white proteins and the gel strength of heat-induced gel.

MATERIALS & METHODS

SIX DRIED EGG WHITE samples were obtained from several suppliers: Ballas (Zanesville, OH), Nutrisearch (Egg City, Moorpark, CA). TEPCO (Jackson, MS), and Walbaum (Wakefield, NE).

Gel preparation

Nine percent egg white solutions were made with distilled water. The solutions were poured into 25 × 52 mm tubes; gel formation was induced by placing the tubes in a 95°C water bath (10 min). Gels were removed from the water bath and cooled to room temperature (25°C) before measurement of gel strength.

Gel strength

Gel strength was measured using a Chatillon Texture Measurement System (J. Chatillon and Sons, Inc., Kew Gardens, NY), and a Lucite probe. The initial clearance between the probe and sample was 2.5 mm; a force setting of 50 kg and a head speed of 1.8 were used (Ehrman, 1988).

−SH Measurement

Protein sulfhydryl groups (−SH) were measured in 1% egg white solutions by the method of Robyt et al. (1971). SDS (0.1%) was added for denatured protein −SH measurements. Protein concentration of the solutions was measured using the Biuret method (Gornall et al., 1949).

Statistical methodology

The correlation coefficient of −SH group concentration and gel strength was determined using a Texas Instruments TI-55-II programmable calculator.

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RESULTS & DISCUSSION

The sulfhydryl group concentration (−SH in 0.1% SDS) of the dried egg white samples ranged from 34.2 to 51.2 μM SH/g. Beveridge and Arntfield (1979) previously reported 51.6 ± 0.5 μM SH/g in fresh egg white (−SH in approximately 0.5% SDS). The differences between the two studies might be due

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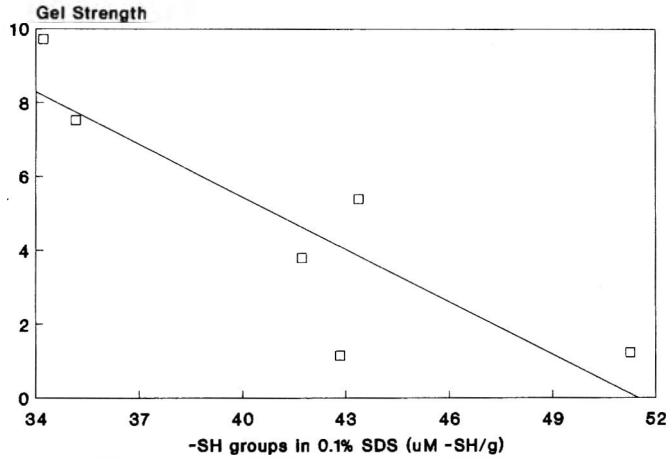

Fig. 1—Egg white −SH groups (in 0.1% SDS) vs egg white gel strength.

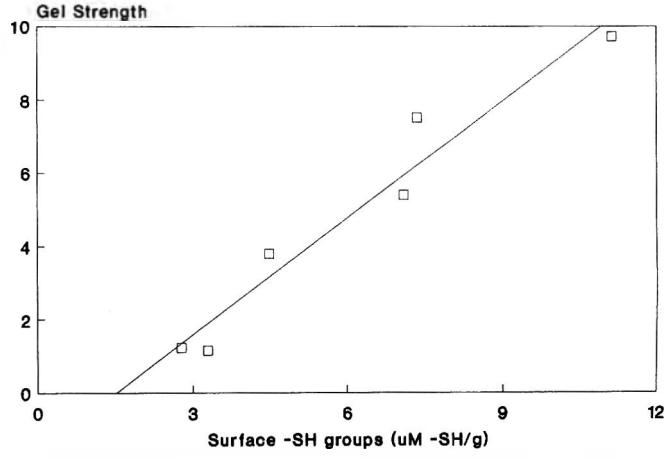

Fig. 2—Egg white surface −SH groups (no SDS) vs egg white gel strength.

A Research Note

Effects of Temperature and Oxygen on the Growth of *Listeria monocytogenes* at pH 4.5

ROBERT L. BUCHANAN and LORI A. KLAWITTER

ABSTRACT

Growth effects were studied using tryptose phosphate broth adjusted with hydrochloric acid. The microorganism survived for extended periods at low incubation temperatures (5 and 10°C), and grew at intermediate temperatures (19 and 28°C). Aerobic incubation at 37°C resulted in relatively rapid inactivation of the organism; however, when oxygen was restricted the organism recovered and survived for extended periods. Oxygen restriction enhanced the growth rate at 19°C. Results demonstrated temperature and oxygen availability interacted to influence survival of *L. monocytogenes* in low pH environment.

INTRODUCTION

TRADITIONALLY, *Listeria monocytogenes* has been considered relatively sensitive to acid environment, being unable to grow at pH <5.5 (Gray and Killinger, 1966). However, several recent studies indicated that, in microbiological media, the organism could grow at initial pH levels as low as 4.4 (Conner et al., 1986; George et al., 1988; Sorrells et al., 1989; Parrish and Higgins, 1989; Petran and Zottola, 1989; Farber et al., 1989; Buchanan and Phillips, 1990). A factor that appears important in regard to growth of *L. monocytogenes* in a low pH environment is the temperature of incubation. Sorrells et al. (1989), Parrish and Higgins (1989), and Farber et al. (1989) reported incubation temperature had a relatively slight but distinct effect on the minimum pH that would support growth of the microorganism. In a more detailed study George et al. (1988) reported the minimum pH that supported growth of *L. monocytogenes* was 4.4, 4.4, 4.6, 4.6, 5.2 at 30, 20, 10, 7, and 4°C, respectively.

While those studies identified an interaction between incubation temperature and minimum pH, they did not assess the effects on growth kinetics. This information is needed to estimate how rapidly and to what extent the organism would grow under such conditions. Further, those studies were largely performed using aerobic conditions. Recent studies in our laboratory (Buchanan et al., 1989; Buchanan and Phillips, 1990) have suggested the organism may more readily tolerate adverse conditions when grown in an oxygen restricted environment. Accordingly, the objective of our study was to determine the effects and interactions of incubation temperature and atmosphere on growth kinetics of *L. monocytogenes* in microbiological media at pH 4.5. This pH was selected, in part, because it was often used as a target pH for control of other pathogens such as *Salmonella* and *Clostridium botulinum*.

METHODS

Bacterium

Listeria monocytogenes Scott A was used throughout the study. Stock cultures were maintained in Brain Heart Infusion (BHI) Broth at 4°C. Starter cultures were grown aerobically on a rotary shaker (150 rpm) in Tryptose Phosphate Broth (pH 7.2) (TPB) (Difco) for 24 hr at 37°C.

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Culture techniques

The bacterium was cultured aerobically and anaerobically using the technique of Buchanan et al. (1989). Briefly, the TPB was adjusted to pH 4.5 using concentrated HCl, and transferred in 50 ml portions to either 250-ml Erlenmeyer flasks (aerobic cultures) or 250-mL trypsinizing flasks (anaerobic cultures). The flasks were sealed with foam plugs or screw caps + rubber septa, respectively, sterilized by autoclaving, and pre-equlibrated to the desired incubation temperature (5, 10, 19, 28, or 37°C). The flasks were inoculated to a target level of 10^3 cfu/ml. The anaerobic cultures were flushed with N_2 10 min, and sealed. This reduced oxygen content of the flasks to 85–145 ppm. All flasks were then incubated at the appropriate temperature on rotary shakers (150 rpm). At least three replicate cultures were followed for each temperature/atmosphere combination.

A limited number of trypsinizing flask cultures were overlayed with 15 mL of presterilized paraffin oil to enhance anaerobiosis. The oil was added immediately after inoculation. These cultures were then flushed with nitrogen and incubated without agitation.

Periodically, 2.5 mL samples were removed from the Erlenmeyer and trypsinizing flasks using a pipette and a hypodermic needle + syringe, respectively. After diluting appropriately with sterile 0.1% peptone water, samples were plated in duplicate on BHI Agar plates using a Spiral Plater (model D, Spiral Systems, Bethesda, MD). All plates were incubated 24 hr at 37°C and enumerated using a Spiral Systems Colony Counter (model 500A). The pH of the 0-hr samples were determined to ensure maintenance of the target pH.

Growth curves

Where appropriate, growth curves were generated using the Gompertz function as described by Buchanan et al. (1989), and used to calculate generation times (GT), exponential growth rates (EGR), lag phase durations (LPD), and maximum population densities (MPD).

RESULTS

AEROBIC GROWTH of *L. monocytogenes* Scott A in TPB at pH 4.5 was highly dependent on incubation temperature (Fig. 1a). Active growth (i.e., increase in population density > 1 log cycle/mL) was observed in conjunction with incubation temperatures of 19 and 28°C. The higher temperature appeared optimal in regard to growth rate; however, a greater maximum population density was observed with 19°C (Table 1). The microorganism did not grow at 5 and 10°C, but survived essentially unchanged for extended periods. *L. monocytogenes* died off relatively rapidly when the incubation temperature was 37°C.

The effect of incubation temperature on growth of *L. monocytogenes* at pH 4.5 in sealed, nitrogen-flushed flasks was similar to the aerobic cultures in regard to overall pattern of response, though differences associated with oxygen availability were apparent (Fig. 1b). Growth was again observed only at 19 and 28°C, with extended survival occurring at 5 and 10°C. The growth kinetics parameters (Table 1) observed with the aerobic and anaerobic 28°C cultures were similar. However, the 19°C anaerobic cultures had decreased LPD's and

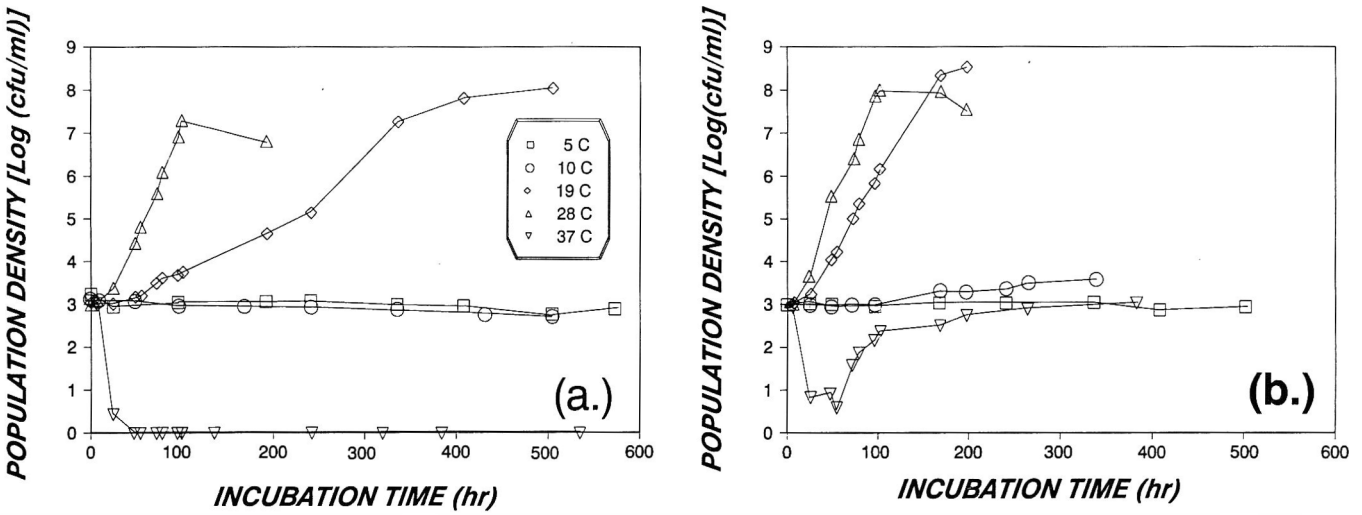

Fig. 1—Effect of incubation temperature on aerobic (a) and anaerobic (b) growth of Listeria monocytogenes *Scott A in tryptose phosphate broth adjusted to pH 4.5. Means of three determinations.*

Table 1—Growth kinetics of cultures that grew in tryptose phosphate broth adjusted to pH 4.5.[a]

Atmosphere	Incubation temp (°C)	Gompertz values A	C	B	M	LPD (hr)	EGR [Log (CFU/mL)/hr]	GT (hr)	MPD Log (CFU/mL)
Aerobic	19	3.0	6.0	0.0072	219.7	80.6	0.016	19.1	9.0
		(0.1)	(0.2)	(0.0004)	(11.6)	(12.6)	(0.001)	(0.4)	(0.2)
	28	3.1	4.1	0.0489	52.4	30.8	0.073	4.1	7.2
		(0.1)	(0.2)	(0.0060)	(3.4)	(1.5)	(0.005)	(0.3)	(0.2)
Anaerobic	19	3.0	6.3	0.0193	79.2	27.3	0.044	6.8	9.2
		(0.1)	(0.2)	(0.0006)	(2.6)	(2.3)	(0.001)	(0.1)	(0.1)
	28	3.0	5.4	0.0376	47.8	21.1	0.074	4.1	8.4
		(0.1)	(0.2)	(0.0031)	(2.1)	(0.9)	(0.007)	(0.4)	(0.2)

[a] Average of three independent determinations; values in parentheses are standard deviations. See text for definitions of abbreviations.

GT's as compared to the aerobic cultures. Again, greater MPD's were observed with the 19°C cultures. The greatest difference between the aerobic and anaerobic cultures was with incubation temperature of 37°C. After an initial decline in population density, two of the three replicate cultures recovered to a level approximating the initial inoculum, remaining at that level for extended periods. The third culture dropped below the limits of detection and did not recover.

The differential response between the aerobic and anaerobic cultures at 37°C was studied further by simultaneously running both aerobic and anaerobic cultures. Included was a third group of cultures consisting of sealed trypsinizing flasks that were overlayed with paraffin oil and incubated without agitation to provide an even more restrictive environment in regard to available oxygen (data not shown). The aerobic cultures again died off. After an initial decline, all of the anaerobic cultures recovered at least to the level of the inoculum. Two of the three cultures overlayed with paraffin oil behaved in a similar manner, while the third did not recover from the initial decline in population density.

DISCUSSION

OUR STUDY extended the recent body of literature indicating that, at least in microbiological media, *L. monocytogenes* could survive and grow at pH 4.5 (George et al., 1988; Sorrells et al., 1989; Ahamad and Marth, 1989; Petran and Zottola, 1989; Parish and Higgins, 1989). The microorganism's response to this low pH was highly dependent on incubation temperature, with survival at low temperatures (5 and 10°C), growth occurring in mid-range (19 and 28°C), and some degree of inactivation at the upper end of the range (37°C). This pattern was similar to that reported by Sorrells et al. (1989); however, a kinetics approach allowed quantitative assessment of the growth at pH 4.5 including the impact of oxygen availability.

Oxygen content had two distinct effects on the microorganism. At 19°C, a growth-permissive temperature, anaerobiosis enhanced growth by decreasing both LPD's and GT's (Table 1). Restricting oxygen content appeared to help the organism overcome the combined adverse effects of nonoptimal pH and temperature since no difference in the kinetics of aerobic and anaerobic cultures was observed at 28°C. The second even more distinct effect was the ability of oxygen to enhance destruction of *L. monocytogenes* when the cells were incubated at 37°C. The initial inactivation was indicative of the combination of low pH and elevated incubation temperature interacting to cause a state of "acid injury" (Buchanan et al., 1988). If oxygen were restricted, in most instances the organism had the ability to repair the physiological lesion(s) associated with acid injury, leading to recovery and subsequent long term survival of the cells. However, in presence of oxygen the lesions were exacerbated, leading to permanent inactivation of the culture. The underlying cause of this oxygen effect was not known. However, it seemed reasonable to hypothesize that one of the physiological lesions resulting from the combination of low pH and elevated temperature was a transient inactivation of enzymes that detoxify active oxygen species such as peroxides and superoxides (Lehninger, 1975). In an anaerobic environment, this would not be a critical lesion, particularly if cell systems could subsequently repair the damage. However, in an aerobic system even the transient inactivation of these enzymes would be fatal as the normal metabolic activity of the cell continued to generate toxic oxygen species. Catalase and superoxide dismutase have been hypothesized to play a role in allowing *L. monocytogenes* to survive adverse intracellular environments (Welch, 1987) and sublethal thermal stress (Dallmier and Martin, 1988).

L. monocytogenes was generally considered to grow optimally at 30–37°C (Rosenow and Marth, 1987; Petran and Zottola, 1989), when other growth factors were optimal. Our results

suggested that *L. monocytogenes* better tolerated adverse conditions when incubation temperatures were somewhat suboptimal. Likewise, though the microorganism was generally considered to grow better aerobically, limiting oxygen appeared to enhance survival and growth when other growth factors were suboptimal. Buchanan and Phillips (1990) also concluded that *L. monocytogenes* was well adapted to microaerophillic environments and that limiting oxygen could enhance its ability to grow under adverse conditions. Identification of the underlying physiological mechanisms responsible for enhanced growth or survivability at suboptimal temperatures and oxygen contents requires future research.

Our study and others (George et al., 1988; Sorrells et al., 1989; Parish and Higgins, 1989; Petran and Zottola, 1989; Buchanan and Phillips, 1990) have indicated that under appropriate conditions *L. monocytogenes* could grow at pH levels as low as 4.4. However, this should be considered a "worst case scenario" in that conditions are optimal for growth and survival of the microorganism. For example, Ryser and Marth (1988) reported that *L. monocytogenes* was unable to grow in cultured or uncultured whey at pH values less than 5.4. Similarly Conner et al. (1986) reported that the organism grew at pH 5.6 but not 4.8 in clarified cabbage juice adjusted with lactic acid. A key factor influencing minimum pH appeared to be the identity of the acid used to alter the pH. Hydrochloric acid, employed in the studies reporting growth of *L. monocytogenes* at pH 4.5, was the least inhibitory to the organism (Sorrells et al., 1989). Organic acids employed as acidulants in foods have been reported to be substantially more detrimental (Sorrells et al., 1989; Ahamad and Marth, 1989; Farber et al., 1989), thereby enhancing control of the bacterium.

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to (1) differences in egg white concentration (0.1 vs 0.5% SDS) or (2) the method used to dry the egg whites. This suggested that drying egg whites might affect the protein conformation and thus the availability of −SH groups.

Comparisons of the −SH group concentrations with the gel strength of a 9% egg white gel are shown in Fig. 1 and 2. Gel strength decreased with increasing −SH group concentration as measured in 0.1% SDS ($r = -0.86$) (Fig. 1). This was the opposite of what would be expected if sulfide bonds (S-S) were formed between the egg white proteins to form a gel. When only the surface −SH group concentration (no SDS) was measured, the gel strength increased with more surface −SH groups ($r = 0.97$) (Fig. 2).

The data indicated that surface −SH groups played an important role in strong heat-induced gel formation in egg whites. It is possible that surface −SH groups can enhance gel network formation by forming S-S bonds with other protein molecules. However, internal −SH groups may form internal S-S bonds that lock the proteins into conformations that make them poor gel formers. Opening the structure of the egg white proteins by drying (perhaps to a limited extent) apparently might promote stronger gel formation due to the higher concentration of surface −SH.

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A Research Note

Physical-Chemical Characteristics of Partially Clarified Guava Juice and Concentrate

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ABSTRACT

Partially clarified guava juice concentrate was prepared from single strength guava puree (5.5°Brix) by treatment with pectinase (2 hr at 50°C), extraction of juice with a rack-and-cloth press, and vacuum concentration to 23°Brix. The concentrate had the following characteristics: density, 1.10; pH, 3.16; total acids, 4.67%; ash, 1.51%; moisture, 72.4%; ascorbic acid, 867 mg%; viscosity, 4.4 cp; and CIE L* 39.2, CIE a* 10.33, CIE b* 27.11.

INTRODUCTION

MARKET DEMANDS for "natural" juices devoid of food additives have prompted food scientists to create clarified, deionized, decolorized, and deodorized juices from new sources. Fractionated juices have been combined with one or more whole juices to obtain sugar/acid ratios, flavors, and colors acceptable to consumers. Thus, the demand for fractionated juices has created another market, especially when made from exotic, tropical fruits.

In Hawaii, guavas are grown for commercial production of purees and nectars; clear juices and concentrates are not manufactured at present. Boyle et al. (1957), Jagtiani et al. (1988) and Brekke (1971) reported methods for producing clarified guava juice and concentrates. However, these earlier reports did not specify many of the physical-chemical characteristics of the raw material or final products.

The objectives of this study were to obtain those physical and chemical characteristics of guava puree, partially clarified single strength and concentrated guava juices, and guava press cake that are needed by food scientists, processors, product formulators, and processing equipment manufacturers in development work.

MATERIALS & METHODS

Processing and sample preparation

Guava puree (*Psidium guajava,* L.) (680 kg) was prepared aseptically and packaged in 18.1 kg "bag-in-box" containers at the Amfac Tropical Products plant in Keaau, Hawaii, employing the method of Chan and Cavaletto (1982). The aseptic puree was shipped by ocean freight to the Horticulture and Processing Division, Department of Scientific and Industrial Research (DSIR), Auckland, New Zealand, where pilot scale juice extraction and concentration studies were conducted.

Pectinex Ultra Sp-L (0.2% w/w, 26,000 PG units, Novo Laboratories, Wilton, CT) was added to each bag of aseptic guava puree which was recapped and incubated in a water bath at 50°C to attain a product temperature of 40°C for 2 hr. Based on the pectin test, we found holding the enzyme-treated puree at 20°C for 16 hr was an acceptable alternative to 50°C for 2 hr and required less handling.

Two percent (w/w) filter flock (Eagle-Picher Industries, Cincinnati, OH) was added to enzymatically treated guava puree and the juice was extracted with a rack-and-cloth filter press. Juice was extracted also directly from enzymatically treated guava puree without added filter aid.

Partially clarified guava juice was concentrated in a Centri-Therm CT-1B evaporator (Alfa-Laval, Lund, Sweden) operating at −0.55 to −0.60 P kg/cm² vacuum at the secondary side (vapor). The concentrate was packed in enameled No. 2 (307 × 409) cans and frozen. Though possible to prepare concentrates of higher °Brix, we decided to use approximately 23°Brix (or 3.9-fold) due to better product quality and economics of operations.

Analytical methods

The pectin test of Waldt and Mahoney (1967) was used to test for completion of enzymatic reaction and additional treatment time was allowed until the reaction was complete.

pH was measured with a glass-electrode pH meter; °Brix was measured with a digital refractometer. Total titratable acidity was determined by the AOAC method no. 22.061 (AOAC, 1980) and the results expressed as percent citric acid. AOAC method no. 22.027 (1984) was used to determine ash content, and density was determined by AOAC method 9.009 (1980) using a pycnometer.

The high pressure liquid chromatography method of Watada (1982) was used to measure ascorbic acid.

AOAC methods 43.A14-43.A20 (1984; supp. no. 1, 1985; supp. no. 2, 1986) were used to determine total dietary fiber.

The alcohol-soluble color (ASC) index and total carotenoids were analyzed as described by Chan and Cavaletto (1982).

Color changes were measured by presenting 100 mL of sample juice in a standard optical juice cell (Hunterlab) in a Hunter Colorimeter (Labscan II). Results were expressed in CIE L*, a*, b* values. Viscosity expressed in sec was measured using AOAC method no. 22.009 (1980) and viscosity expressed as cp was determined with a Brookfield viscometer Model LVF at 60 RPM.

Statistical analysis

Statistical analyses were performed using SAS version 5.0 (SAS Institute Inc., 1985). The General Linear Model (GLM) Procedure was employed. All significant differences are reported at the 95% confidence level. Measurements were performed in quadruplicate, except for pilot plant experiments which were done in duplicate.

RESULTS & DISCUSSION

FOR ENZYMATICALLY TREATED purees at 50°C for 2 hr, a slight decrease in juice yield was observed compared to those treated at 20°C for 16 hr but juice with more desirable pinkish tint, which remained even after concentration, was obtained.

Fruit pressing yielded juice containing 0.6% suspended solids. For aseptically packed guava puree treated with pectinase and pressed with filter aid, juice yields increased significantly ($p<0.05$) by 8.5% for those samples treated at 20°C for 16 hr and 10.0% at 50°C for 2 hr.

Single strength guava juice was concentrated to about 4-fold; i.e., 3.91-fold concentration in °Brix, 4.03-fold in %total

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Table 1—Characteristics of aseptic guava puree[a], partially clarified single strength and concentrated guava juices, and guava press cake

	Guava puree		Guava juice		Guava press cake
	Control	With enzyme	Single strength	(3.9x) Conc	
Density, g/mL	1.035	1.042	1.013	1.103	–
SE	0.005	–	0.007	–	–
°Brix	6.25	6.19	6.09	23.81	6.00
SE	0.08	0.05	0.05	0.05	0
pH	3.28	3.12	3.10	3.16	3.75
SE	0.07	0.01	0.01	0.13	0
Instrumental color					
CIE L*	52.32	51.81	32.30	39.02	54.37
SE	0.02	0.01	0.01	0.02	0
CIE a*	24.03	23.09	0.03	10.33	20.17
SE	0.01	0.02	0.01	0.02	0
CIE b*	18.16	18.42	11.80	27.11	30.54
SE	0.01	0.02	0.02	0.04	0
Ash	0.30	–	0.36	1.51	0.45
SE	0.00	–	0.01	0.01	0.01
% Moisture	93.2	–	93.9	72.4	74.9
SE	0.00	–	0.10	0.23	0.06
Alcohol soluble color of juice					
browning (415 nm)	0.071	0.074	0.046	0.092	–
haze (440 nm)	0.034	0.035	0.023	0.049	–
Viscosity					
Relative, sec	9.93	1.56	1.38	1.28	–
SE	0.17	0.01	0.02	0.02	–
Brookfield, cp	1310	140	3.5	4.4	–
SE	12.00	0.85	0.41	0.51	–
% total acidity (as citric acid)	1.09	1.17	1.16	4.67	1.11
SE	0.001	0.001	0.013	0.260	0.013
Ascorbic acid, mg/100 g	206	209	207	867	–
SE	0.50	1.00	0.50	1.00	–
Total dietary fiber, %	–	–	–	–	49.00
SE	–	–	–	–	0.20
Total carotenoids, mg/100 g	–	–	–	–	52.7
SE	–	–	–	–	0.42

[a] Aseptic guava puree was enzymatically treated (pectinase) at 50°C 2 hr (product temp 40°C). 2% (w/w) filter flock used during juice extraction with a rack-and-cloth filter press.

titratable acid, 4.18-fold in ascorbic acid, and 4.19-fold in ash (Table 1). The relatively high recovery of ascorbic acid after concentration agrees with previously obtained results with papaya puree concentrates evaporated also with a Centri-Therm CT-1B evaporator (Chan et al., 1975). Addition of the enzyme resulted in slight decreases in CIE L* (brightness) and "a*" (redness) values, but this effect was compensated by slight increases in "b*" (yellowness) value, resulting in a duller pink color. Alcohol soluble color indices as browning were not affected. However, an approximate 10-fold reduction in viscosity allowed for easier product handling, increased juice yield and higher extraction efficiency.

Before concentration, single strength guava juice color was similar to that of apple juice diluted with about 33% water but with a pink hue. After concentration, the guava juice had a browner color in spite of a slight increase in "brightness" ("L*"), due to perceptible increases in both "yellowness" ("b*") and "redness" ("a*"). This color darkening was indicated also by a 2-fold increase in alcohol soluble color indices, another measure of browning. An increase in concentration did not cause viscosity to increase significantly because the pectinases already had effectively hydrolyzed the viscous pectins. This lessened any further difficulty in product handling and equipment operation as was reported previously by Brekke et al. (1978).

Guava press cake contained about the same amount of soluble solids in serum as did single strength guava juice, and acidity was comparable to that of single strength guava puree. Moisture content was similar to that of the concentrated juice. In comparison to puree, guava press cake had a darker pink color. The Hunter color measurement showed higher "yellowness" ("b*") and "lightness" ("L*") values and slightly lower "redness" ("a*"). The perceived darker pink color may have been due to the greater reflectance from a solid material (press cake) compared to that of a fluid substance (puree). Total carotenoid content was 52.67 mg/100g. Guava press cake had a total dietary fiber content of 49%, which may make it a suitable bulking agent.

Except for a "watery" mouthfeel, also shown by an 84% decrease in relative viscosity (Table 1), enzymatically treated guava puree retained the same desirable characteristics in color, aroma, and flavor as the untreated puree. Single strength juice retained some original pink color in the puree while most of the color remained in the press cake. Concentrated guava juice had a darker and browner juice color than single strength juice. Guava aroma noted in the single strength juice and press cake was scarcely detectable in the concentrate. Single strength and concentrated juices had a tart, guava flavor with a clean aftertaste that is desirable in formulated products. Guava press cake, which retained a very mild guava flavor, may be used to increase perceived "body" in formulated juices. Other uses for press cake, such as a possible source of fruit fiber, should be studied.

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A Research Note

Alginate Texturization of Highly Acid Fruit Pulps and Juices

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ABSTRACT

Apple pulp and reconstituted grapefruit juice were provided as texturized alginate gels at concentrations of 5–96%, after pH adjustment. The resulting products had appreciable mechanical integrity even at very high pulp or juice concentration. Their texture, judged by compressive strength, strain at failure, and deformability modulus was improved considerably by dipping in calcium lactate solution. The original pH of the fruit could be restored by a dip in a citric acid solution without adverse effects on texture.

INTRODUCTION

THE TECHNOLOGY of fruit analog preparation from alginate gels has been known for many years (Luh et al., 1976; Hannigan, 1983; Tolstoguzuv and Braudo, 1983; Glicksman, 1983), and is covered in several patents (Szczesniak, 1968; Wood 1975). The gel strength, as well as other rheological properties, is usually regulated by adjusting the gum concentration. In the practical range, with algin as with other gums, increasing algin concentration results in increased stiffness, deformability and strength. Thus it offers higher resistance to deformation, can sustain considerable deformation before rupture and fails at higher stress.

In contrast, addition of fruit pulp tends to weaken the gel. That is an especially acute problem when the pulp has high acidity and a low pH, on the order of 3–3.5. In such cases, products with more than about 10% pulp are so weak they collapse under their own weight (Nussinovitch and Peleg, 1989). From a nutritional and marketing viewpoint, there is a clear advantage to products that contain a portion of fruit that is several times higher than the limiting 10%. To make such products, the standard procedures of gel preparation need to be modified.

The objectives of this work were to develop a modified procedure to prepare products containing up to 90% fruit pulp, and to assess their rheological properties. The performance of this modified procedure was tested with commercial apple pulp and reconstituted grapefruit juice (pH $\simeq$ 3.5) that could not be texturized at concentrations higher than about 10% by a conventional method using only alginate.

MATERIALS & METHODS

Texturization

Additives of free applesauce (Mott's, 13° Brix) and frozen concentrated grapefruit juice (Stop and Shop, a local supermarket chain, 37° Brix) both of low pH (pH = 3.4–3.5) were used as raw materials. Food grade commercial alginate powder (Kelgin, LV, Kelco Division of Merck & Co.) was used for cold set alginate-fruit gel preparations. The apple pulp and grapefruit concentrate were neutralized with 2M sodium hydroxide, and the total soluble solids content was adjusted to 10° Brix with distilled water. Alginate and calcium hydrogen orthophosphate ($CaHPO_4$) powders were mixed and added together to neutralized fruit pulp or juice to which water had previously been added. The fruit pulp or juice content was controlled by the amount of water added at this stage. The concentration of fruit pulp or juice in the alginate solution varied between 0–96%. After about 1 hr of mixing, a freshly prepared solution of glucono - δ - lactone was added. Concentrations were alginate 1.5% (wt), $CaHPO_4$ 1.0% (wt), and glucono - δ - lactone 1.5% (wt) on the basis of the gel solution before setting.

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Sample preparation

Cylindrical gel specimens of (1.7 cm × 1.5 cm) were prepared as described by Nussinovitch et al. (1989). They were aged 48 hr before mechanical testing. After aging some gel specimens were immersed an additional 48 hr in 2% calcium lactate, 0.1–5% citric acid and 0.1% citric acid + 2% calcium lactate solutions to restore acidity and improve texture. Since syneresis was observed in all gels, during aging and/or dipping the exact dimensions of each specimen were determined separately with a caliper.

Mechanical testing

The product specimens were compressed to failure between parallel lubricated plates at a deformation rate of 10 mm/min. using an Instron UTM with a 50 kg cell. The Instron was interfaced with a Macintosh II computer through a Strawberry Tree interface card. Special computer software was used for machine control and data acquisition, as well as for mechanical analysis. This included conversion of the output voltage vs time relationships to digitized corrected (true) stress (σ_{cor}) vs Hencky's strain (ϵ_H) files using the following definitions:

$$\sigma_{cor} = \frac{F(t)\,[H_o - \Delta H(t)]}{A_o H_o} \quad (1)$$

$$\epsilon_H = \ln\left[\frac{H_o}{H_o - \Delta H(t)}\right] \quad (2)$$

where, H_o was the initial specimen length, $\Delta H(t)$ the momentary absolute deformation, and A_o the cross-sectional area of the original specimen. The strength (stress at failure) and the strain at failure were reported in stress and strain terms as defined by Eq. (2) and (3), respectively.

The deformability modulus, ϵ_D, was determined as the slope of the linear portion of the stress-strain relationship, i.e.

$$E_D = \frac{\sigma_{cor}}{\epsilon_H} \quad (3)$$

The strain range at which the stress-strain relationship was linear was determined by testing the regression coefficient, r^2, at different strains.

All mechanical tests were performed in duplicate or triplicate and results were reported as mean values.

RESULTS & DISCUSSION

THE pH of the "raw" gel, before final acidification, was in the range 4.1–4.5 for the apple pulp and 4.3–5 for the grapefruit juice. These pH levels were significantly lower than those of the neutralized pulp juice and were a result of acid released by the glucono - δ - lactone. They were not only significant in quantitative terms but also from a product safety view point. They demonstrated the possibility, at least in principle, to texturize the product at high pH but "age" it in an acid environment (see below). We should also add that duration of the

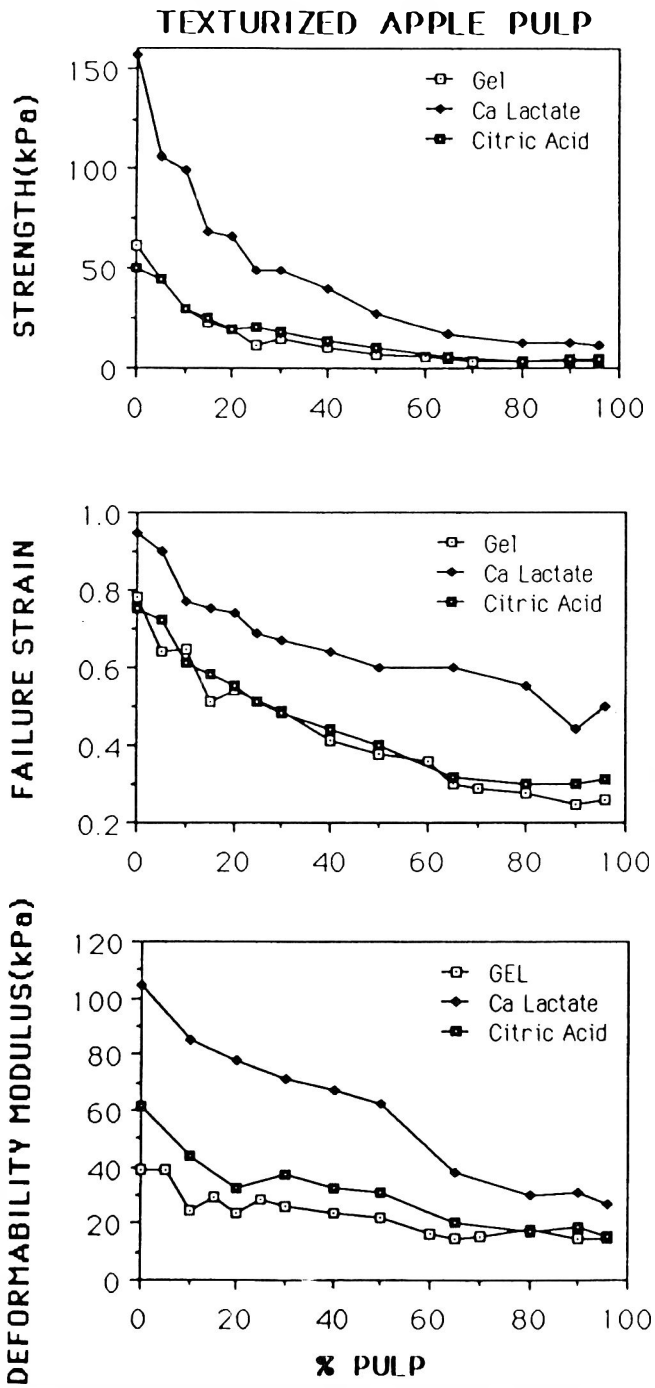

Fig. 1—Mechanical properties of texturized apple pulp products as a function of pulp concentration.

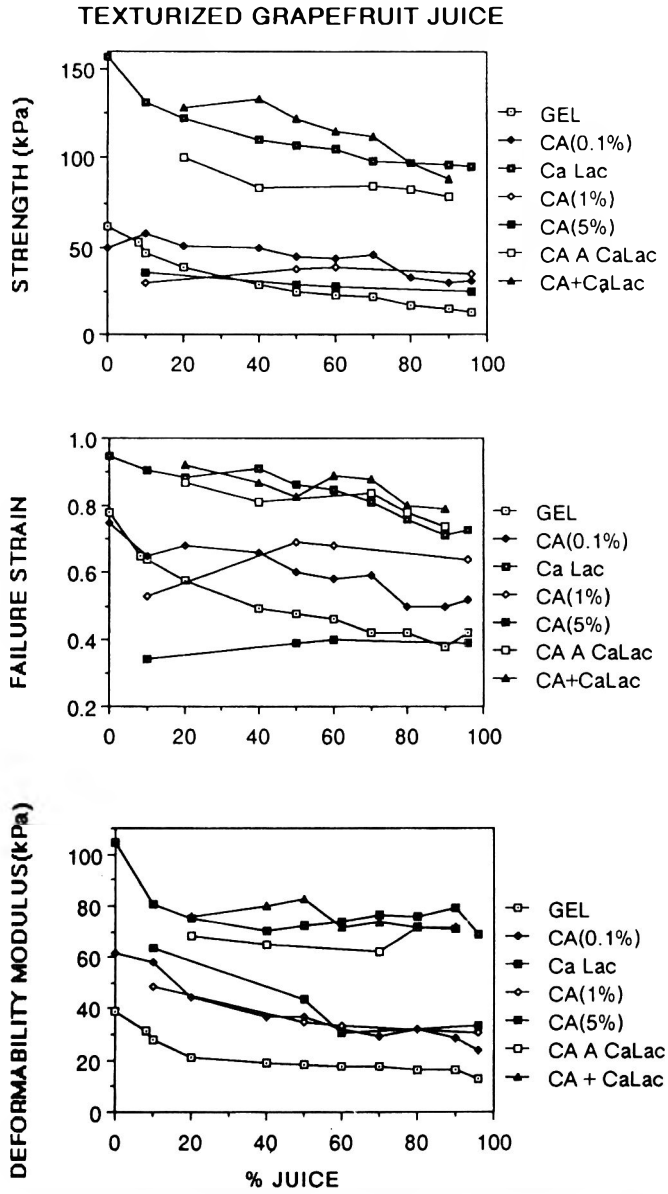

Fig. 2—Mechanical properties of texturized grapefruit juice products as a function of juice concentration. CA A CaLac — dip in a calcium lactate solution and subsequently in citric acid. CA + CaLac — dip in mixed solution of citric acid and calcium lactate.

texturization stage was particularly long in this work. This was because the product was molded to enable meaningful mechanical testing. In an industrial process, similar products could be prepared in much shorter time.

After the acid dip the pH further dropped to 3.2–3.9 in the acid baths or remained about the same in the calcium lactate baths (4.5–5.4). The overall shrinkage in the process, a result of syneresis of alginate based gels, was about 49% in the pure gel (no fruit) and declined to 9% and 10% in the apple pulp and grapefruit juice products containing 96% fruit.

Since the main objective of our work was to test the possibility of preparing mechanically stable products containing high pulp or juice content, no attempt was made to fine tune the process with respect to product pH at each stage using a buffer or other methods. Clearly the pH of the final product could easily be controlled by adjusting the acid bath concentration and exposure time. Furthermore, since alginate gels are a thermoset product, microbial stability can be achieved by heat treatment as well as by chemical preservatives which might be less desirable.

Mechanical properties of texturized apple pulp

The compressive strength (failure stress), strain at failure and deformability modulus of texturized apple pulp as a function of pulp concentration are shown in Fig. 1. Although the strength, deformability and stiffness decreased considerably as the pulp concentration increased, the products, even with 50–96% pulp had appreciable mechanical integrity. In contrast, apple products prepared without pH adjustment could not be considered solid at concentrations above 10%. As could be expected, a dip in calcium lactate solution resulted in a firmer gel irrespective of pulp concentration. This was evident by an increase in all three mechanical parameters, that is, by a factor of 2.3–4.4, 1.2–2 and 1.8–3.5 in strength, deformability and

stiffness, respectively. The dip in a dilute citric acid bath (0.1%) restored the pH to a level of 3.2–3.4 but had only marginal effect on mechanical behavior of the products (Fig. 1).

Mechanical properties of texturized grapefruit juice

With texturized grapefruit (Fig. 2) as in the case of texturized apple pulp, pH adjustment resulted in products with appreciable mechanical integrity even with juice concentrations at more than 90%. Although, as before, increasing fruit content resulted in a weaker gel, the effect was not as drastic as with apple pulp. In comparison to texturized apple pulp, the effect of calcium lactate on the grapefruit gels was much stronger as judged by the increase in mechanical parameters, that is, a 2.5–7.3, 1.2–1.9 and 2.6–5.3 fold increase in compressive strength, deformability and stiffness, respectively. Acid dip not only restored pH to the 3.2–3.9 level but also improved firmness. The effect, however, was much milder than that of calcium lactate dip, i.e. up to 2.3 fold increase in strength and 1.6–2.1 in stiffness.

The effect of calcium lactate was also separately studied in the grapefruit products by changing exposure conditions. Thus, the gels were also dipped in a mixture of citric acid and calcium lactate or alternatively in acid bath first and then calcium lactate solution. The results are also shown in Fig. 2. Evidently the firm structure, irrespective of pulp contents, was primarily due to exposure to calcium ions. The manner by which pH was adjusted, that is whether calcium lactate was added together with acid or after the acid treatment, did not appear significant.

The texturized grapefruit products were also exposed to citric acid at different concentrations. Unlike the previous results, the effect on the mechanical parameters did not increase or decrease in unison. Furthermore, while the dip in 5% acid always increased stiffness, as judged by the deformability modulus, it could have a diminishing effect on their strength and deformability, especially at low pulp. Dip in 1% citric acid solution seemed to be an effective means to increase deformability of products with high juice content with only minor effects on strength, as compared with 0.1% acid.

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A Research Note

Enzymatic and Color Changes During Post-harvest Storage of Lychee Fruit

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ABSTRACT

Hunter color dimension scores were related to loss of red color and darkening during post-harvest storage of fresh lychee fruit at 4°C. Peroxidase declined whereas polyphenyloxidase increased in the lychee endocarp during storage. The peroxidase of the exocarp increased to 36.93 U/min at 29 days, but declined during subsequent storage. No change in polyphenyloxidase of the exocarp was observed until 29 days when activity increased nearly ten fold to 0.55 U/min. The decrease in peroxidase and increase in polyphenyloxidase activities coincided with onset of discoloration of fruit.

INTRODUCTION

POST-HARVEST BROWNING precludes widespread commercial marketing of lychee fruit (*Litchi chinensis* Sonn.). Akamine (1960) reported that ascorbic acid, citric acid, sulfur dioxide fumes and dips, fruit waxes or vinyl resin plastic coatings did not effectively preserve red color. Blanching fruit in water between 56°C and 100°C inhibited browning onset, but also bleached anthocyanins (Akamine, 1960). Post-harvest treatment with hot 0.05% benomyl dip at 52°C for 1 min with an overwrap in polyethylene bags (Scott et al., 1982) or polyvinyl chloride (Huang and Scott, 1985) minimized browning. Dehydration of the fruit accelerated browning (Akamine, 1960; Campbell, 1959). Storage of fruit in perforated, polyethylene bags at 2°C to 8°C at 65–95% relative humidity maintained the color as long as 5 wk but mold and decay developed after 3 wk storage (Campbell, 1959). Microbial spoilage of fruit was inhibited by treatment with 2% sodium hypochlorite and extended shelf life by 1 wk when stored in perforated polyethylene bags near 7°C and 85% relative humidity (Gaur and Bajpai, 1978).

Browning of fruit tissue is usually associated with enhanced enzymatic activity, especially peroxidase (POD) and polyphenyloxidase (PPO) (Joslyn and Ponting, 1951). Joubert and van Lelyveld (1975) reported that PPO and POD activities in lychee were higher in bruised fruit. The objectives of our study were to determine if activity of PPO and POD changed during post-harvest storage and if changes in activity coincided with browing discoloration.

METHODOLOGY

Sample preparation

Lychee fruit was donated by J.R. Brooks and Sons (Homestead, FL). Fruit were harvested, wrapped in polyethylene bags, stored in 2.3 kg lots, transported to the Citrus Research and Education Center, and stored at 4°C. For enzyme analysis, about eight fruit were used for each replication for each sampling period. The exocarp was separated from the endocarp and each sample was homogenized at 4°C in four volumes of 50 mM potassium phosphate, 1M KCl, 2% polyvinylpolypyrolidone (PVP), pH 6.2. The homogenate was centrifuged at 16,000 × *g*, 30 min, 4°C and the filtered supernatant constituted the crude enzyme extract.

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Enzyme assays

Peroxidase activity was measured according to the method of Nagle and Haard (1975). Polyphenyloxidase was measured according to the method of Flurkey and Jen (1978). One unit of peroxidase or polyphenyloxidase activity was defined as the increase of 0.1 unit of absorbance per min at 470 or 420 nm, respectively.

Hunter L*a*b* color dimensions

Hunter L*a*b* color dimensions were measured in the reflectance mode using a HunterLab D25-PC2 tristimulus colorimeter (Hunter Assoc., Inc. Reston, VA) equipped with a D25 A optical sensor and PC computer. Individual whole fruit were coded and the same fruit were analyzed in the apex position throughout the study.

Values of reported enzyme activity and color dimensions were the average of triplicate analyses of three and ten observations, respectively. Analysis of variance and Duncan's Multiple Range Test were used for least significant difference at the 0.05% level.

RESULTS & DISCUSSION

Hunter color dimensions

Color analyses of stored lychee fruit are shown in Table 1. The Hunter L values continually decreased with storage time, indicating that the fruit became darker. L values decreased significantly on the eighth day. Similarly, Hunter a and b values significantly decreased between 0 and 12 days. This decrease in Hunter a and b values implied that stored lychee had less red and less yellow characteristics. The saturation index (SI), which indicated the relative amount of color remaining in the lychee fruit, decreased significantly with storage time. Hue, as represented by hue angle, gradually increased significantly with storage time. This corresponds to a visible image changing to a darker yellow upon storage. Such color deterioration was consistent with a loss of red color and browning during storage. Storage times of fruit used to determine Hunter color dimensions were notably shorter than for fruit used for enzymatic analysis and may reflect accelerated deterioration due to increased handling and warming at room temperature during color measurements.

Enzyme activity

Changes in peroxidase and polyphenyloxidase of the endocarp and exocarp are indicated in Table 2. The POD activity of the endocarp declined from an initial 13.06 U/min to a final 3.77 U/min after 48 days. The POD activity of the exocarp increased significantly from an initial 17.58 U/min to 36.76 U/min at 15 days and remained relatively constant through 29 days. A significant decline in activity was observed between 29 and 36 days storage and continued to a final value of 9.99 U/min at 48 days.

No significant change in PPO activity of the endocarp was observed until an increase in activity at 29 days. The PPO

*Table 1—Changes in Hunter color dimensions during post-harvest storage of lychee fruit**

Time (Days)	L	a	b	SI	Hue Angle	ΔE
0	22.98[a]	26.10[a]	8.58[ab]	27.49[a]	18.24[d]	0.00[e]
2	22.76[ab]	24.93[ab]	8.44[ab]	26.33[ab]	18.76[d]	2.66[d]
6	22.29[abc]	26.12[a]	8.67[a]	27.54[a]	18.48[d]	3.07[d]
8	22.07[bcd]	24.42[abc]	8.49[ab]	25.87[bc]	19.25[cd]	3.72[d]
12	21.62[cde]	23.72[bc]	8.21[abc]	25.11[bc]	19.14[cd]	3.72[da]
14	21.44[de]	22.98[c]	8.16[bc]	24.39[c]	19.60[bcd]	4.24[d]
16	20.93[e]	21.11[d]	7.90[c]	22.56[d]	20.65[bcd]	6.04[c]
20	20.07[f]	20.46[d]	7.34[d]	21.75[d]	19.89[bcd]	6.91[c]
22	19.73[f]	20.26[d]	7.24[d]	21.53[d]	19.96[bcd]	7.44[c]
26	19.53[fg]	16.25[e]	6.79[e]	17.89[e]	27.71[a]	11.65[ab]
28	18.91[gh]	15.43[ef]	6.60[e]	17.66[e]	23.38[bc]	11.01[b]
30	18.25[h]	14.33[f]	6.18[f]	15.63[f]	23.82[ab]	13.03[a]

[a-h] Means with the same letter within the same column are not significantly different. Alpha = 0.05.

* Color dimensions representing value, hue, chroma, hue angle and saturation index were calculated as described by Little (1975): $L^* = 10^*Y^{1/2}$, $a^* = 17.5\ (1.02X-Y)/Y^{1/2}$, $b = 7.0(Y-0.847Z)/Y^{1/2}$, saturation index = $SI = (a^2 + b^2)^{1/2}$, hue angle = $\tan^{-1}$ (b/a). Total color difference between individual samples at zero day and the indicated storage time was calculated as described by Hunter (1975): $\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$.

*Table 2—Changes in peroxidase and polyphenyloxidase activities in the endocarp and exocarp during post-harvest storage of lychee**

TIME (Days)	POD-ENDO	POD-EXO	PPO-ENDO	PPO-EXO
0	13.06[a]	17.58[c]	0.02[d]	0.04[d]
2	9.74[b]	22.76[bc]	0.02[d]	0.03[d]
8	9.76[b]	27.88[b]	0.02[d]	0.02[d]
15	9.12[bc]	36.76[a]	0.03[cd]	0.04[d]
29	8.72[bc]	34.62[a]	0.04[c]	0.04[d]
36	6.06[d]	25.62[b]	0.08[b]	0.55[b]
39	5.31[de]	26.10[b]	0.08[b]	0.85[a]
42	7.85[c]	19.46[c]	0.08[b]	0.27[cd]
44	4.24[e]	20.06[c]	0.09[b]	0.56[b]
48	3.77[e]	9.99[d]	0.18[a]	0.45[bc]

* POD-endo and POD-exo denotes peroxidase in endocarp and exocarp, respectively. PPO-endo and PPO-exo denotes polyphenyloxidase in endocarp and exocarp, respectively. One unit = increase of 0.1 unit absorbance per min.

[a-e] Means within same column with different letters not significantly different at 0.05% level.

activity continued to increase to a value of 0.18 U/min at 48 days. The PPO activity of the lychee exocarp did not change significantly from an initial 0.04 U/min through 29 days storage. At least a ten fold increase in PPO activity to 0.55 U/min was observed at 36 days storage and the activity remained elevated throughout subsequent storage.

Nearly a twofold increase in exo-POD was observed between 0 and 29 days storage, whereas endo-POD, endo-PPO, and exo-PPO changed little. A two- to threefold decrease in endo- and exo-POD and a 10- to 20-fold increase in exo-PPO activity was observed between 29 and 48 days storage. Onset of fruit discoloration was subjectively judged to begin at day 29 and to increase throughout subsequent storage. The changes in enzymatic activities suggest that exo-POD activity may be an early indicator of darkening propensity during storage and may possibly serve as a marker enzyme for lychee quality. The changes in endo-POD and exo-PPO were more dramatic during longer storage. While earlier research inferred oxidative enzymes were involved in darkening (Akamine, 1960; Joubert and Van Lelyveld, 1975), our study showed a definitive role of enzymatic oxidation in post-harvest darkening. Use of barrier polymers such as perforated polyethylene bags (Akamine, 1960; Scott et al., 1982) or polyvinyl chloride (Huang and Scott, 1985) reduced the rate of respiration and subsequent accumulation of phenolic precursors. Use of barrier packaging with a higher CO_2/O_2 permeability in conjunction with O_2 scavengers (Kader et al., 1989) and oxidase inhibitors could conceivably more effectively extend post-harvest shelf life of lychee.

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A Research Note

Dilatancy in Starch Solutions Under Low Acid Aseptic Processing Conditions

ROBERT V. DAIL and JAMES F. STEFFE

ABSTRACT

A rheological characterization of 2.72, 3.14, and 3.59% (g dry starch/100g water) waxy maize starch solutions was conducted at 121.1°C. The two highest concentrations are increases over those previously examined. Flow behavior indexes ranged from 1.1 to 1.9 in this study, and results indicate the shear-thickening behavior observed was due to dilatancy instead of time-dependent thickening caused by on-going pasting.

INTRODUCTION

IN CONTINUOUS STERILIZATION of viscous liquid foods using high temperature short time methods (aseptic processing), the food flows through a tube at the required temperature until sterilization is achieved. To design the tube length, rheological properties of the food are required so the velocity profile, and consequently the residence time of the fastest moving element, can be determined. Rheological properties are temperature sensitive, so they must be determined at actual process temperatures (typically 121–143°C for low acid foods during aseptic processing).

In previous work (Dail and Steffe, 1990), cross-linked waxy maize starch solutions were rheologically characterized under low acid aseptic processing conditions. Dilatancy (shear-thickening) was observed in 22 of 23 experiments. The best explanation for this observation was that small shear stresses in the fluid, from high temperatures, low shear rates, and low starch concentrations, could not deform the relatively rigid starch granules. This follows the same logic used by Bagley and Christianson (1982), Christianson et al. (1982), and Christianson and Bagley (1983) for their dilatancy observations.

Other factors which may have contributed to shear-thickening, such as ongoing pasting and/or different gelatinization rates were considered but thought to be insignificant: Short residence time in the viscometer should have minimized the ongoing pasting effect, and we thought varying rheological properties caused by different gelatinization rates observed in other studies were due to use of starch with a significant amylose fraction.

Shear-thickening caused by small shear stresses that do not deform rigid starch granules constitutes true dilatancy. "Shear-thickening" caused by ongoing pasting is time-dependency and could be termed "apparent" dilatancy. This should be distinguishable from true dilatancy by increasing shear stress either by lowering temperature, increasing concentration, or increasing shear rate. If the flow behavior index (n) decreased with increasing concentration beyond a critical concentration, the dilatancy would be true. Alternatively, if "shear-thickening" increased with starch concentration, it may be due to time-dependency caused by ongoing pasting. Increasing shear stress by lowering temperature was not an option, since rheological parameters determined below 121°C would be of little use for design of aseptic thermal processes. Also, there was little capacity to increase shear rate, because the heat exchanger could not heat the solutions to 121°C at flow rates higher than those studied previously.

Consequently, the objective of this research was to verify true dilatancy in the fluids previously studied (Dail and Steffe, 1990) by determining rheological parameters for the same starch at greater concentrations. This would also provide additional rheological parameters for design of aseptic thermal processes for low-acid foods.

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MATERIALS & METHODS

THE TUBE VISCOMETER SYSTEM, operation, and data collection procedures were detailed previously (Dail and Steffe, 1990). The same starch (National 465, lot# KH 4929, National Starch and Chemical Corp., Bridgewater, NJ) was used. The lot number differed, because the previous lot number was no longer available. This is a cross linked waxy maize starch recommended for thickening retorted or aseptically processed low acid foods. Three samples were dried in a moisture oven at 103°C for 24 hrs. Moisture content was 10.24%.

The experimental design was a single factor (concentration), randomized complete block design. Three concentrations (2.72, 3.14, and 3.59%) were examined (based on g dry starch/100g water). Randomization was within a block, so each block was a replication. Four blocks were executed (one/day). Blocking over time insured randomization of any day to day unwanted experimental or systematic effects. Experiments were conducted at 121°C.

The lowest concentration here equals the highest studied in the previous work. This was done to examine the effect of using different lot numbers. The highest concentration represents an upper limit for the tube viscometer system. At higher concentrations, temperature control became difficult due to increased viscosity. Also, the heat exchanger could not heat higher concentrations to 121°C and maintain the highest flow rate due to the increased energy required to gelatinize the starch. All solutions were made with tap water due to the large amount required for each experiment. Each batch was tested for pH.

RESULTS & DISCUSSION

PARAMETER ESTIMATES for the two parameter power law model ($\tau = K\gamma^n$) were determined (Table 1). The block four experiment at the highest concentration failed. It could not be repeated, because more starch from the same lot was not available. Fitting data from the three experiments at the highest concentration with the three parameter Herschel-Bulkley model showed an insignificant yield stress. Tap water average pH was 7.66 ± 0.36 (95% confidence limits).

Table 1—Values of the consistency coefficient, K (Pa s^n), and flow behavior index, n (dimensionless), from each experiment. Each block is a replication of three concentration levels

%Starch	Block 1	Block 2	Block 3	Block 4
2.72%	$K = 6.27*10^{-5}$ $n = 1.719$	$K = 3.10*10^{-5}$ $n = 1.851$	$K = 2.59*10^{-5}$ $n = 1.389$	$K = 3.60*10^{-5}$ $n = 1.840$
3.14%	$K = 2.53*10^{-4}$ $n = 1.416$	$K = 2.16*10^{-4}$ $n = 1.436$	$K = 8.08*10^{-4}$ $n = 1.118$	$K = 1.97*10^{-4}$ $n = 1.475$
3.59%	$K = 1.84*10^{-4}$ $n = 1.587$	$K = 3.93*10^{-5}$ $n = 1.896$	$K = 5.06*10^{-4}$ $n = 1.394$	failed

Table 2—Treatment means, and parameter values estimated from pooled data, for each concentration

Starch Concentration	Treatment Means	Pooled Data Estimates
2.72%	$K = 3.60*10^{-5}$	$K = 2.89*10^{-5}$
	$n = 1.840^{a}$	$n = 1.872$
3.14%	$K = 3.69*10^{-4}$	$K = 2.58*10^{-4}$
	$n = 1.361^{b}$	$n = 1.398$
3.59%	$K = 2.43*10^{-4}$	$K = 1.433*10^{-4}$
	$n = 1.626^{a,b}$	$n = 1.643$

[a,b] Means followed by same letter not significantly different at P = 0.01.

The estimated values (Table 1) of the consistency coefficient (K) and flow behavior index (n) were each treated as a single response for a particular experiment. Analysis of variance examined block and treatment effects for both parameters. Block effects were not significant (P = 0.16 for K and 0.24 for n) indicating changes in either parameter were not due to unwanted experimental or systematic effects. Consequently, the analyses were rerun examining treatment effects alone. Since the only factor in the experimental design was concentration, these analyses were an examination of concentration effects, which on K were not significant (P = 0.15). This means changes in K were not necessarily attributable to concentration change. Concentration effects on n were significant at $\alpha = 0.05$ (P = 0.012).

Insignificant block effects allow averaging parameters for a given concentration or estimating parameters from pooled data at a given concentration. Treatment means and pooled estimates were determined (Table 2). They were nearly the same, which was expected. Parameters estimated from pooled data are more desirable than treatment means for a given concentration (Beck and Arnold, 1977).

The average values of the rheological parameters for 2.72% starch in the present work are different from those determined previously at 121°C ($K = 2.60*10^{-4}$ and n = 1.558). The current values, however, are within two standard deviations of the previous values. The pH in each study was the same, so these differences are probably due to (starch) lot to lot variations. This is disturbing, from a process control standpoint, because of the effect rheological properties have on hold tube velocity profiles.

Duncan's New Multiple Range Test applied to the mean values of n (Table 2) support the hypothesis that the shear thickening observed previously (Dail and Steffe, 1990) was true dilatancy: there was a significant decrease in n as the starch concentration was increased from 2.72 to 3.14%. However, the support is not strong, because the value of n at 3.59% was not significantly different from the two other values. Further delineation requires a stronger experimental design with more replications and/or higher concentrations.

In the previous work (Dail and Steffe, 1990), the parameters (K and n) were shown to be almost correlated by near linear dependence of sensitivity coefficients (Beck and Arnold, 1977). Also, plots of sensitivity coefficients showed n was dominant. The relationship between K and n approached the following function:

$$K = \exp(-4.534n - 1.647)$$

The parameter values in the current study approached that same relationship. Consequently, as before, behavior and changes in K are explained more by near correlation of parameters than by physical reasons such as concentration changes. For more detail, see Dail and Steffe (1990).

CONCLUSIONS

THE HYPOTHESIS that aqueous solutions of modified corn starch show true dilatancy under low acid aseptic processing conditions is supported by a significant decrease in the flow behavior index when starch concentration increased from 2.72 to 3.14%. A broader experimental design, with more replications and/or higher starch concentrations, is needed for more conclusive results. The parameter values are helpful for design of aseptic thermal processes for low acid foods.

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A Research Note

Kinetic Study on Color Changes in Milk Due to Heat

ELLA PAGLIARINI, MONICA VERNILE, and CLAUDIO PERI

ABSTRACT

Analysis of changes in terms of color difference (ΔE) and yellowness index (YI) permits evaluation of severity of heat treatment of milk. Both ΔE and YI increased at constant temperature following zero-order kinetics, at temperatures between 90 and 130°C for 1 to 60 min. Activation energy, according to the Arrhenius equation, was 101.8 and 117.5 KJ/mol for ΔE and YI, respectively. Comparison of instrumental and sensory data showed a minimum ΔE of 3.8 would be attained before visual perception of milk browning. Color values, such as ΔE or YI, are proposed as the simplest and most reproducible indices of commercial milk quality.

INTRODUCTION

COLOR CHANGES, mainly due to Maillard reactions, occur in milk following heat treatments. Quantitative evaluation of brown compounds may be considered an indicator of severity of heat treatments (Mauron, 1981). Starting from the work of Kessler and Fink (1986), this correlation has been investigated systematically using increasingly sensitive and reliable tristimulus colorimeters. Color data by means of such procedures may, for example, contribute to distinctions between sterilized and UHT milk (Andrews and Morant, 1987; Fink and Kessler, 1988). Activation energy of the browning reaction, according to Kessler and Fink (1986), was 116 KJ/mol.

Recently, Rhim et al. (1988a) evaluated tristimulus Hunter L-, a- and b-values of milk subjected to heat treatments over a wide range of temperatures (100–150°C) and times (0.5–180 min). According to their results, Hunter L-values decreased following first-order kinetics (Ea = 114.8 KJ/mol) and Hunter a- and b-values increased following zero-order kinetics (E_a = 104.9 and 110.3 KJ/mol, respectively). The Browning Index, also (reflectance ratio of 520/430), correlated with milk browning effect. Its value increased following zero-order kinetics (E_a = 123.5 KJ/mol).

Our work presents data from heat-treated milk at temperatures between 90 and 130°C. Our objective was to evaluate browning reactions in terms of ΔE and YI, and also visually in order to identify threshold sensory perception of color changes.

MATERIALS & METHODS

Milk was heat treated in stainless steel capillary tubes having 2 mm internal diameter and 1 mm wall thickness. Tubes 2.0m long, corresponding to holding volume about 7 mL, were spiral-coiled for a compact, easy-to-handle shape. They had short straight portions and screw plugs at both ends. The capillaries, because of their high surface-to-volume ratio (3:1) and small diameter, were considered highly efficient heat transfer devices, allowing rapid, homogeneous temperature increases in the milk, without significant overheating. The tubes, filled with fresh skim milk and perfectly sealed with screw plugs were dipped into an oil bath previously set at the selected temperature. At the end of the experiment the tubes were immediately transferred into a water-ice bath for cooling. The time was precisely measured from the instant of dipping in the oil bath to the instant of dipping in the ice water. This procedure was justified by the assumption that heating-up and cooling-down times were negligible in comparison with total holding time. Direct measurement of milk temperature, by introducing a T thermocouple (±0.2°C) into a test capillary at oil bath temperatures between 70 and 140°C, showed heating times between 3.8 and 4.1 sec. These times were considered as negligible in treatments lasting 60 sec or longer. The heat-treated samples, after cooling, were recovered by simply unscrewing the plugs and pouring out the milk.

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Instrumental assessment of milk color

The color of each milk sample was determined using a colorimeter (Chromameter II Reflectance, Minolta Camera Co., Ltd, Japan). A white tile no. 101947 was used to standardize the instrument.

Four mL of milk were pipetted into a glass capsule (2 cm diam × 3 cm ht) with a thick layer of black teflon wrapped around the side to produce an opaque reflective surface. The colorimeter measured the values L, a and b of the CIELAB color difference equation developed from the Hunter system (Francis and Clydesdale, 1975). The difference of color (ΔE) between samples and raw milk reference was determined by the following equation:

$$\Delta E = \sqrt{\Delta a^2 + \Delta b^2 + \Delta L^2}$$

Yellowness Index (YI) was expressed as (Francis and Clydesdale, 1975):

$$YI = 142.86\ b/L$$

Visual assessment of milk color

Milk samples were evaluated by sensory tests to identify the visual perception threshold of browning. A simple triangle comparison test was carried out between treated and raw milk by a trained panel of 10 members. This evaluation was performed by daylight; each sample was placed in white, 3 cm high, plastic dishes on a gray surface.

RESULTS & DISCUSSION

FIGURE 1 shows ΔE variation vs time at various temperatures. Data in Fig. 1 demonstrate browning of milk follows zero-order kinetics. However, we noted that the straight lines did not start from the origin of the coordinates but have dif-

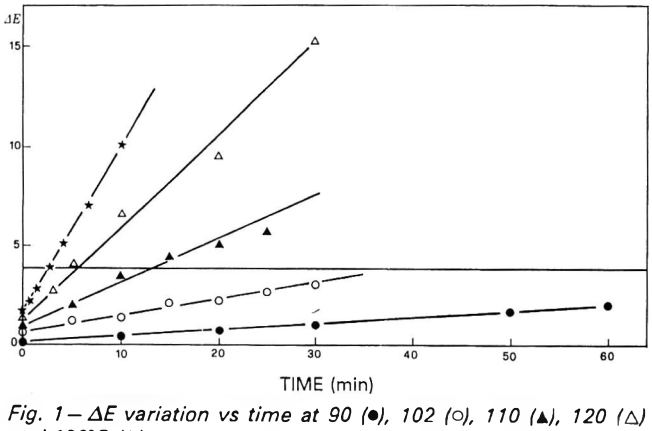

Fig. 1 – ΔE variation vs time at 90 (●), 102 (○), 110 (▲), 120 (△) and 130°C (★).

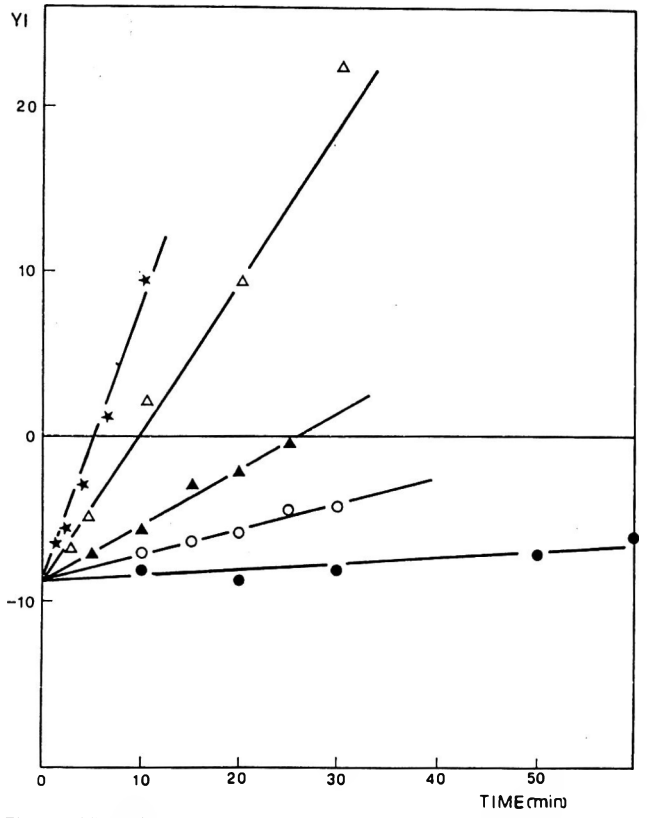

Fig. 2—YI variation vs time at 90 (●), 102 (○), 110 (▲), 120 (△) and 130°C (★).

ferent intercepts: the higher the treatment temperature, the higher the intercept.

This phenomenon was observed by others (Burton, 1955, 1984; Giangiacomo and Messina, 1988; Rhim et al., 1988b). It derives from a sharp initial increase of the Hunter L-parameter due to the whitening effect caused by denaturation of serum proteins. Browning kinetics is described by the following equation:

$$\Delta E = \Delta Eo + kt \quad (1)$$

where ΔEo represents the intercept varying with heating temperature, k is the rate constant and t is the time.
ΔEo can be expressed as:

$$\Delta Eo = -13.8909 + 0.0385785\ T \quad (2)$$

where T is the heating temperature in k.

The rate constant value can be calculated according to the Arrhenius equation as follows:

$$k = ko\ e^{-Ea/RT} \quad (3)$$

$$k = 1.5 * 10^{-13}\ e^{-101.8/8.314 * 10^{-3}T} \quad (4)$$

The ΔE data, at each heating temperature, fit very well to Eq. (1) as shown in Fig. 1, where both experimental points and calculated straight lines are reported.

The activation energy value calculated from our data was 101.8 KJ/mol. This value was similar, though slightly lower, than that obtained by Kessler and Fink (1986) and by Rhim et al. (1988a).

Comparison of sensory and instrumental data demonstrated the visual perception threshold of browning corresponded to a ΔE value of 3.8 (horizontal line in Fig. 1). This value represented a lower browning level as perception threshold than that reported by Kessler and Fink (1986). Plotting our data according to the Kessler and Fink procedure we found the line of perception threshold reported by Kessler and Fink as $F^* = 1$ approximates $\Delta E = 5$.

Figure 2 shows variations of the Yellowness Index (YI) vs time at various temperatures. Each line started from the same intercept and the reaction followed zero-order kinetics. This relationship can be expressed as:

$$YI = YIo + kt \quad (5)$$

where: $YIo = -8.8$, $k = ko\ e^{-Ea/RT} = 5.75 * 10^{13} * e^{-117.5/8.314 * 10^{-3}T}$,

T = absolute temperature, t = time in min.

Figure 2 showed very good agreement between experimental points and calculated lines (correlation coefficients between 0.87 and 0.99). The activation energy of the YI calculated according to the Arrhenius equation was 117.5 KJ/mol, practically identical to that obtained by Kessler and Fink (1986).

In conclusion, both ΔE and YI are sensitive heat-damage indices and the calculated equations permit browning effects caused by heat treatments of milk to be predicted satisfactorily.

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