JOURNAL OF FOOD SCIENCE

6 November-December 1995
Volume 60, No. 6

Coden: JFDAZ 60:1159-1402
ISSN: 0022-1147

Journal of Food Science & IFT Information

Basic Research, Applied Science and Engineering

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 JOURNAL OF FOOD SCIENCE (formerly *Food Research*) is published six times a year (bimonthly) by Institute of Food Technologists, 221 N. LaSalle Street, Suite 300, Chicago, IL. 60601 USA. Printed in the USA. Second class postage paid at Chicago, Illinois and at additional mailing offices. POSTMASTER: Send address changes to *Journal of Food Science*, 221 N. LaSalle St., Suite 300, Chicago, IL 60601.

Memo *FROM THE SCIENTIFIC EDITOR*

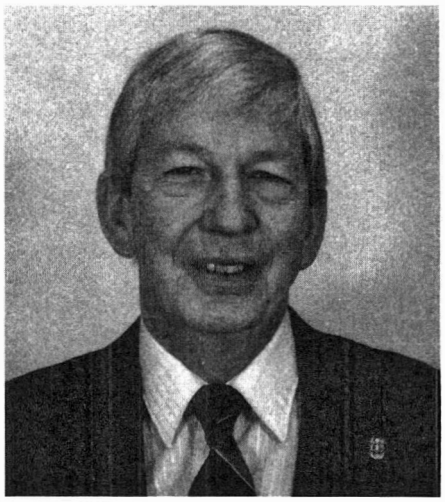
Looking into the Future

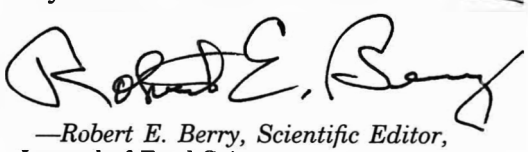
We've started some innovations this year, the most significant of which has been the initiation of special Feature Articles. We have headlined one in each issue and they seem to be successful. We've been getting good comments from you, our readers, about them and we welcome your submissions of new ones for consideration. We have yet to publish one of the "Forum"-type papers but hope you will give it consideration. These were envisioned to cover reports on subjects where several hypotheses, viewpoints, or interpretations have been proposed. The general problem area would be reviewed, followed by discourses from several researchers relating different viewpoints. Subjects often covered in Symposia or "Hot Topic" reviews at the IFT Annual Meeting may be good prospects for "Forum" reports. Please keep them in your thoughts and send us some proposals. We strongly urge those chairing Symposia or Forums to give this special consideration.

So what else is new? You're going to see more changes in our Journal during the coming year. At the Fall 1995 IFT Committee meetings it was proposed to initiate a new cover design, in color, next year. Not only will this be a cosmetic improvement, but we are going to make it a functional improvement as well. We plan to begin grouping articles in the Table of Contents according to scientific discipline, with brief headings. These will include Chemistry/Biochemistry, Engineering/Processing, Microbiology, and Sensory/Nutrition which will also include the few Foodservice reports we publish. Within these groupings the papers will also be subgrouped according to food commodity, e.g. fruits, vegetables, meats, dairy, seafood, etc. After a trial period we may find it advisable to alter these category groupings but, it will be a start. We think it will be helpful to you readers and we welcome your suggestions.

Other proposals include the publication of JFS Titles, and eventually Abstracts, on the Internet. We saw some exciting demonstrations of CD-ROM potential for publishing past issues of JFS in this readily searchable, speedy, electronic format. The wheels are turning to initiate these and other changes in the near future. If you have suggestions of changes that would make our publications more useful to you and your colleagues, contact the Chair of the Subcommittee on Publications or our Director of Publications and let them know.

Once again, we owe our gratitude to you who have served so graciously and capably as peer reviewers for our published articles. We value the scientific integrity and professionalism with which you have carried out your reviews. You are the principal reasons for the high level of scientific quality and merit of the articles we publish. We recognize you on the following list for your help this year. We hope we have included all of you who helped and we sincerely apologize for any we may have omitted.





—Robert E. Berry, Scientific Editor, Journal of Food Science

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Letters

Texture Profile Analysis— Methodology Interpretation Clarified

As the originator, with my group at the General Foods Technical Center, of the Texture Profile Analysis (TPA) in the 60s I feel very gratified that the method is currently enjoying a renewed interest and popular application. Unfortunately, a mistake has crept into the methodology as presently practiced which I would like to correct and bring to the attention of the editors and researchers using the method.

TPA is based on the recognition of texture as a multi-parameter attribute and on my classification of textural characteristics (*J. Food Sci.* 28: 385-389, 1963). In that classification the mechanical aspects of texture were divided into the primary characteristics of hardness, cohesiveness, springiness, adhesiveness, and viscosity, and the secondary characteristics of brittleness, chewiness, and gumminess. **Chewiness** was defined as the energy required to masticate a **solid food product** to a state of readiness for swallowing. **Gumminess** was defined as the energy required to disintegrate a **semisolid food product** to a state of readiness for swallowing. Instrumentally, chewiness was quantified as a product of hardness × cohesiveness × springiness, and gumminess as a product of hardness × cohesiveness (semisolid products undergo permanent deformation and have no springiness).

Instrumental TPA was developed and its original promise demonstrated using an apparatus simulating chewing—the General Foods Texturometer (*J. Food Sci.* 28: 390-396, 1963). It was not commercially available at the time. When a few years later its production started in Japan, problems developed with import and servicing in the U.S.

TPA became a practical method of instrumental texture evaluation owing to its adaptation to the Instron by my friend and colleague, Dr.. Malcolm C. Bourne of Cornell University. Prof. Bourne was well familiar with the TPA having spent a sabbatical leave in my laboratory during which he studied the mechanisms of action of the Instron and the Texturometer and compared the TPA values they generated. Unfortunately, in his publication which became the key reference to TPA [*Food Technol.* 32(7): 62-66, 72 (1978)] Prof. Bourne inadvertantly failed to make clear that chewiness refers to solid and gumminess to semisolid foods. Contributing to the misunderstanding was his definition of chewiness as "the product of gumminess × springiness." It should be understood that the same product cannot exhibit both chewiness and gumminess, unless as a solid it becomes a semisolid during sensory mastication. Such a transition is practically never accomplished during instrumental TPA evaluation.

Thus, it is incorrect to quantify and report chewiness and gumminess in TPA of solid or semisolid products. Chewiness should be reported for solids and gumminess for semisolids.

—*Alina Surmacka Szczesniak, Mt. Vernon, NY. IFT Fellow, retired; formerly, Principal Scientist, General Foods Technical Center, Tarrytown, NY.*

In support of Methodology Clarification

I thank Dr. Szczesniak for pointing out a misleading statement that appeared in my paper describing instrumental texture profile analysis in the July 1978 issue of *Food Technology.*

She is quite correct that gumminess refers to semisolid foods and chewiness to solid foods and therefore chewiness and gumminess should not both be reported for the same product.

The confusion partly arises from the fact that gumminess is the product of two primary texture characteristics (hardness × cohesiveness) while chewiness is the product of three primary characteristics (hardness × cohesiveness × springiness) two of which are the same as for gumminess. Unfortunately my 1978 paper failed to point out that gumminess and chewiness are mutually exclusive even though they have much in common.

I hope Dr. Szczesniak's letter will clear up this error and I welcome this opportunity to support the clarification she has made.

—*Malcolm C. Bourne, Professor of Food Science & Technology, Cornell University, Institute of Food Science, New York State Agricultural Experiment Station, Geneva, NY 14456-0462.*

A Critical Review

Cholesterol Oxides in Foods of Animal Origin

P. PANIANGVAIT, A.J. KING, A.D. JONES, and B.G. GERMAN

ABSTRACT

The earliest discovery of an oxidation product of cholesterol from a natural source was reported in 1940. Additional discoveries of cholesterol oxidation products (COPs) in edible food products accelerated in the 1980s as gas chromatography and high performance liquid chromatography detection methods were improved. COPs have been found in several foods including liquid eggs and dried egg products, milk and milk products, meat and meat products, marine food products, and other processed foods. Compelling evidence demonstrates that several cholesterol oxides are cytotoxic, atherogenic, mutagenic, and carcinogenic. Therefore, the presence of COPs in foods raises questions about the safety of consumption of some products.

Key Words: toxicity, cholesterol oxides, eggs, milk, meat, marine foods

INTRODUCTION

THE EFFECTS of cholesterol-containing foods in the human diet has been the subject of many investigations in recent years, largely due to the hypothesized link between cholesterol and coronary heart disease (CHD). Although there has been no direct evidence showing cholesterol cytotoxicity, potent angiotoxic effects have been noted for several cholesterol oxides which led researchers to hypothesize a likely role for them in CHD. Results of several investigations demonstrated that 25-hydroxycholesterol and cholestane-3β,5α,6β-triol (cholestanetriol) were the most toxic agents. Therefore, the presence of cholesterol oxidation products reported in certain types of foods raises questions about the safety of cholesterol oxides. Still, many questions remain to be answered before any firm conclusions can be reached regarding the atherogenicity of cholesterol oxides in the diet.

To gain more information about the effects of cholesterol and its oxides, it is necessary to isolate and identify them accurately. Muscle foods are the major source of cholesterol in the diet. Several sterol oxides have been detected in foodstuffs by various methods. However, some quantitative data may not be consistent due to difficulties associated with the separation and analysis of sterol oxides. We developed a comprehensive compilation of data on the content of cholesterol oxides (COPs) in foods of animal origin. We have also included an overview of cholesterol oxidation, biological effects of cholesterol oxides, and various problems associated with accurate and precise quantification of cholesterol and its oxides.

Author King is with the Dept. of Avian Science, author Jones is with the Facility for Advanced Instrumentation, and author German is with the Dept. of Food Science & Technology, Univ. of California, Davis, CA 95616. Author Paniangvait, formerly with the Dept. of Food Science & Technology, is now affiliated with Thai President Foods Public Co., Ltd., Bangkapi, Bangkok, Thailand, 10240.

AUTOXIDATION OF CHOLESTEROL

Initiation

Cholesterol is a molecule with an unsaturated or double bond (Fig. 1); therefore, it is prone to oxidation. It is sensitive to free radical oxidation by diatomic molecular oxygen (O_2) in the air. The initiation process whereby cholesterol reacts with ground state O_2 remains unclear; however, Smith (1981, 1987) has discussed several possibilities.

Physical condition

Reports (Bergström and Wintersteiner, 1941; Smith et al., 1967; Smith, 1981, 1987) indicate that the physical state of cholesterol greatly influences the types of oxidation products. Many different (≈74) oxidation products have been identified or inferred; many remain unidentified, particularly more polar compounds (Smith, 1981).

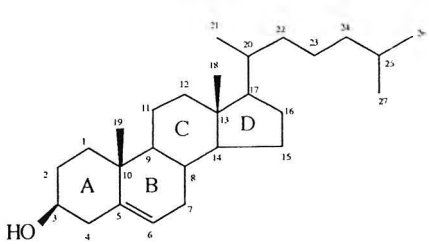

Fig. 1—Cholesterol molecule.

The autoxidation of solid cholesterol has been extensively studied and is well documented (Smith, 1981, 1987). When cholesterol is in the crystalline state and in the presence of air, the oxidation reaction is governed by the arrangement of molecules in the crystal. Cholesterol molecules are arranged in double layers with the 3-hydroxyl groups in juxtaposition and side chains exposed (Smith, 1987).

Cholesterol molecules function as an integral part of the lipid bilayer of cell membrane and are closely associated

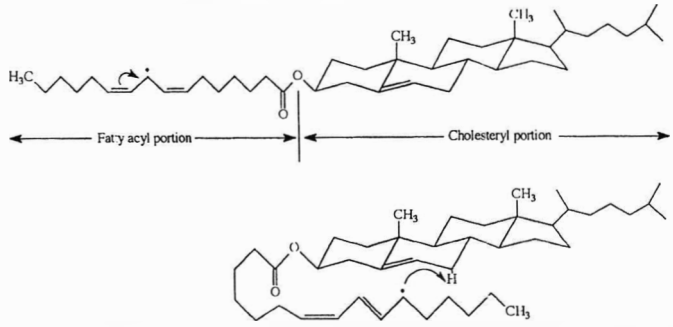

Fig. 2—Suggested intramolecular oxidation of cholesterol ester molecule at the most probable position.

with membrane phospholipids. As cholesterol contains one Δ^5-double bond, formation of any oxygen radical or free radical is expected to initiate cholesterol oxidation (Smith, 1981). Smith suggested that the hydroperoxides of polyunsaturated fatty acids formed during lipid oxidation may be necessary to initiate cholesterol oxidation. The high polyunsaturated fatty acid content of phospholipids in food and their considerable vulnerability to attack by oxidizing species generated within cells and close to the cell membrane have led to the probability that lipid oxidation is initiated at the subcellular membrane level (Igene et al., 1979). It is conceivable, therefore, that cholesterol oxidation should proceed in a way analogous to fatty acid oxidation. Smith (1981) postulated that cholesterol oxidation in food and biological systems may be intermolecular or intramolecular. In intermolecular systems, hydrogen is extracted from cholesterol by peroxy or oxy radicals of oxidized neighboring polyunsaturated fatty acids (phospholipids) in the membrane. In intramolecular systems, the oxidized fatty acyl portion attacks the cholesteryl portion of the same cholesteryl ester molecule (Fig. 2).

The autoxidation of cholesterol fatty acyl esters involves the same radical processes as that of cholesterol (Sevilla et al., 1986) but at a markedly different rate. The autoxidation of cholesterol in aqueous alkali is much faster than that of cholesterol fatty acyl esters (FAE), but autoxidation of FAE is faster if the environment includes heating in air or dissolution in oil (Smith, 1981). The common oxysterols: epimeric 7-hydroxycholesterol; 7-ketocholesterol, epimeric 5,6-epoxycholesterol, and cholestanetriol (esterified) are products of the cholesteryl ester oxidation. Oxidation at C20 and C25 of the cholesterol FAE are also possibilities (Korahani et al., 1982).

The amount of cholesterol remaining after heating with and without different triglycerides at 100°C for 24 hr showed that when cholesterol was heated alone it was practically stable (Osada et al., 1993a, b). However, when it was heated with triglycerides, there were variable degrees of decomposition. When cholesterol was heated with relatively saturated fats, like tristearin and beef tallow, oxidized cholesterol was produced after long-term heating. Polyunsaturated fat (soybean oil, linseed oil, safflower oil, sardine oil, and triolein) oxidized more rapidly than saturated fat (beef tallow and tristearin). When heating with unsaturated fat, in particular with sardine oil, cholesterol was readily decomposed even in 1 hr of heating. The most predominant oxidized cholesterol detected was 7-ketocholesterol, as well as 5β-epoxycholesterol and 5α-epoxycholesterol. Very small amounts of 7α-hydroxycholesterol, 7β-hydroxycholesterol, and cholestanetriol were produced. Osada et al. (1993b) indicated that, possibly, cholesterol in foods is oxidized when it exists together with fats, even with relatively saturated fats.

Triglycerides (tristearin, triolein, trilinolein, and milk fat) accelerated the decomposition of cholesterol at 130° and 180°C (Kim and Nawar, 1991; Nawar et al., 1991); the effect was most marked with triolein. This observation was similar to that of Osada et al. (1993a, b). Such results reflect the instability of unsaturated fatty acids to oxidation at high temperature.

Park and Addis (1985a) also concluded that cholesterol was quite stable. However, if the samples had been under harsh conditions capable of inducing dehydration (freeze-drying or spray-drying), cholesterol oxidation increased. That is, the dehydrated products were more reactive. Fresh bovine brain, liver or muscle did not contain any of the oxides tested, whereas the dehydrated health foods in tablet form contained from 13.8 ppm (7-ketocholesterol) to 46.1 ppm (7α-hydroxycholesterol).

Many studies demonstrated that the amount of COPs in foods can frequently reach 1% of total cholesterol and occasionally 10% or more (Kumar and Singhal, 1992). In model systems cholesterol can be oxidized up to 70% of the original concentration (Bergström and Wintersteiner, 1941; Kimura et al., 1979). Both groups reported that an aqueous cholesterol suspension arrived at an apparent final state where consumption of cholesterol and formation of COPs stopped after more that 70% of the initial substrate was consumed. They suggested that accumulation of COPs in the reaction media may have changed the micelle structure, resulting in cessation of cholesterol oxidation.

BIOLOGICAL EFFECTS OF CHOLESTEROL OXIDES

Effects of cholesterol oxides in atherosclerosis

Cholesterol in the American diet has been indicated as contributing to the high death rate from cancer and coronary heart disease (CHD). The possible association between dietary intake of 5,6α-epoxy-5α-cholestan-3β-ol and development of cancer has caused considerable concern among food scientists, although results were from only one cytotoxicity study and the AMES test for mutagenicity of the epoxides was negative (Ansari et al., 1978). The carcinogenicity of cholesterol epoxides has also been implicated in a few toxicological studies (Ansari et al., 1978; Sevanian and Peterson, 1984).

Although cancer is a disease of profound human concern, atherosclerosis is more directly related to cholesterol, and its effect on initiating the disease has been controversial. Several researchers have focused on the association between cholesterol and atherosclerosis (Grundy, 1986; Ross, 1986; Allred et al., 1990; Kumar and Singhal, 1991). Epidemiological data suggested a relationship between serum cholesterol level and CHD (Grundy, 1990). Animal studies showed a positive correlation between hypercholesterolemia and atherosclerosis (Chaikoff et al., 1948; Faggiotto and Ross, 1984; Higley et al., 1986b).

Chaikoff et al. (1948) fed 2% USP grade cholesterol to chickens to induce hypercholesterolemia and found lesions (atheromata) in the thoracic aorta after 6.5 mo. However, Peng and Taylor

(1984) suggested that the subsequent lesions were likely caused by the impurities as a result of oxidation rather than the USP grade cholesterol used. Higley et al. (1986b) performed feeding trials with unoxidized cholesterol and reported that COPs induced fewer arterial lesions in rabbits than did cholesterol. However, Addis and Park (1989) in reviewing the work of Higley et al. (1986b) noted that (1) the oxides fed to rabbits included high levels of epoxides and low levels (2.0%) of cholestane-3β,5α,6β-triol, generally recognized as the most toxic COPs, (2) investigators did not measure the initiation phase of atherosclerosis where the effect of potent oxides is thought to be most critical and (3) mass spectrometry was not used for ultimate identification of the oxides.

In contrast to the work of Higley et al. (1986b), Hodis et al. (1991) noted a different effect for cholesterol (1%) in the diet of 6-wk-old New Zealand white rabbits. Plasma and aortic tissues from test and control animals were examined for cholesterol and COPs by gas chromatography and mass spectrometry. They found higher quantities of cholest-5-ene-3β, 7α-diol; cholesta-3,5-dien-7-one; 5,6-α-epoxy-5α-cholestan-3α-ol; cholest-5-ene-3β,7β-diol; and 5α-cholestane-3β,5,6β-triol in tissues of test animals when compared to the control. 5,6β-epoxy-5α-cholestan-3β-ol; cholest-5-ene-3β-25-diol, and 3β-hydroxycholest-5-ene-7-one were found in tissues of rabbits fed cholesterol but not in that of test animals. Hodis et al. (1991) concluded that a basal level of COPs found in normocholesterolemic rabbits was elevated by increased consumption of cholesterol. Furthermore, they noted that free radical attack on exogenous cholesterol resulted in the accumulation of oxides in tissue.

Table 1—Common and systematic names of some sterols and cholesterol oxides

Common name	Systematic name	Alternate name/Abbreviated name
cholesterol	cholest-5-en-3β-ol	
7a-hydroxycholesterol	cholest-5-en-3β,7α-diol	7α-OH
7β-hydroxycholesterol	cholest-5-en-3β,7β-diol	7β-OH
7-ketocholesterol	3β-hydroxycholest-5-en-7-one	7-keto
α-epoxide	5α,6α-epoxy-5-cholestan-3β-ol	cholesterol-5α,6α-epoxide/α-ep
β-epoxide	5β,6β-epoxy-5-cholestan-3β-ol	cholesterol-5β,6β-epoxide/β-ep
19-hydroxycholesterol	cholest-5-en-3β,19-diol	19-OH
25-hydroxycholesterol	cholest-5-en-3β,25-diol	25-OH
cholestanetriol	5α-cholestane-3β,5,6β-triol	3β,5α,6β-trihydroxycholesterol/triol

Several investigations demonstrated that 25-hydroxycholesterol and cholestanetriol are toxic agents linked to atherosclerosis (Taylor et al., 1979; Peng and Taylor, 1984; Peng et al., 1991). One toxic COP, 25-hydroxycholesterol, is absorbed in mammals when included in the diet and causes defects in the aortic surface as revealed by scanning electron microscopy (Peng et al., 1982). Various hydroxylated cholesterol derivatives are potent inhibitors of HMG-CoA reductase in the aortic cells to some extent (Peng et al., 1979; Kumar and Singhal, 1991; Lund and Björkhem, 1994). Inhibition of cholesterol biosynthesis by these compounds may cause

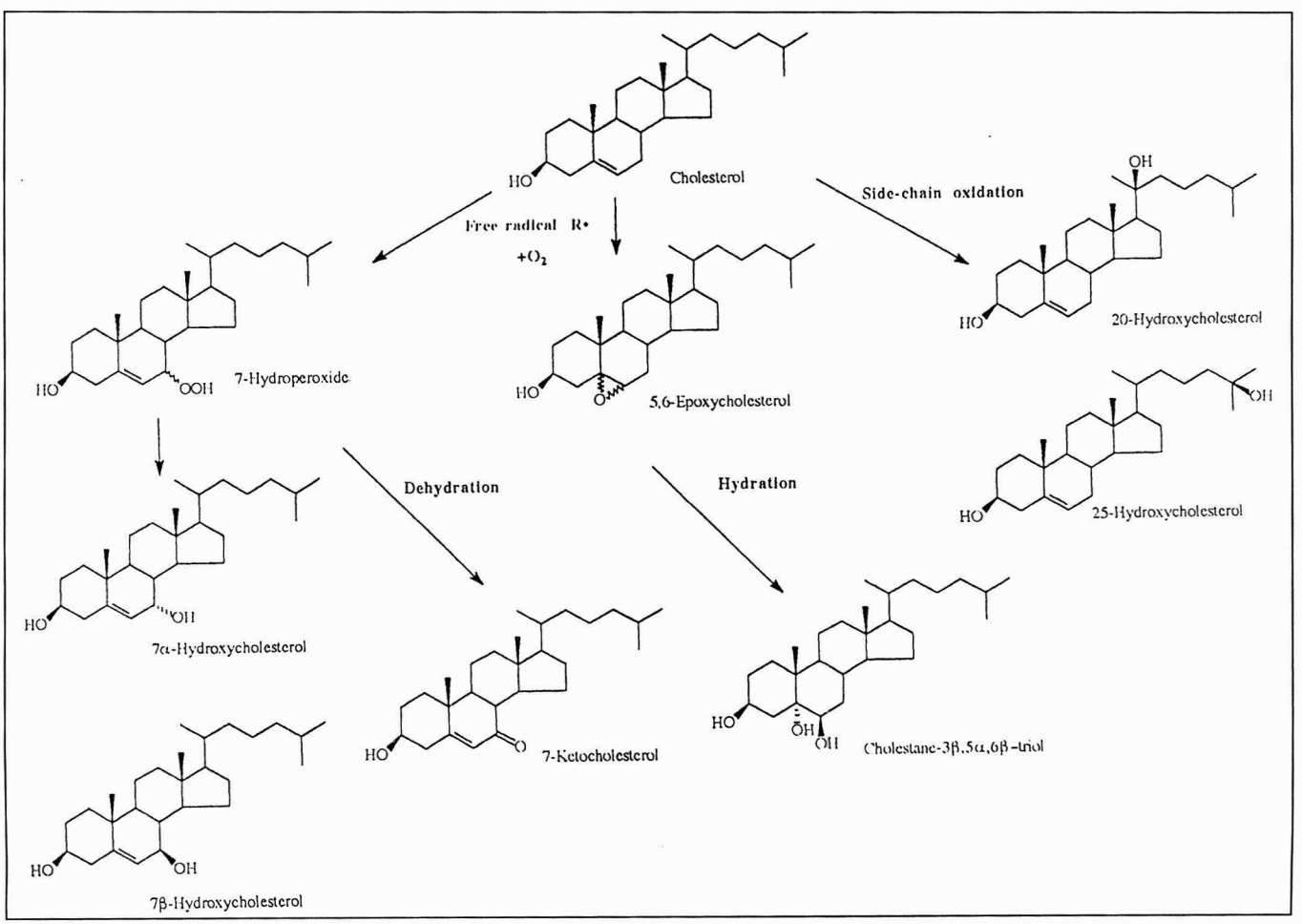

Fig. 3—Common cholesterol oxidation pathways.

cell death because of membrane dysfunction (Peng et al., 1979; Gibbons, 1983; Ghiselli et al., 1992). These dead cells could be the primary entry for lipid infiltration which ultimately led to atherosclerosis (Morin and Peng, 1989; Peng et al., 1991).

Although some researchers have shown that hypercholesterolemia is associated with atherosclerosis, rarely have they identified the involvement of pure cholesterol in the etiological process. Yet, oxidized cholesterol, principally LDL, is implicated in the etiology of atherosclerosis (Grundy, 1986, 1990). According to Grundy, the following steps explain the effects of COPs in atherosclerosis. Circulating particles of LDL are entrapped in the intima as they pass through the endothelium of the arterial wall and into the intimal layer. Entrapment is caused by the association of apoB-100 of LDL with glycoaminoglycans. LDL, and thus cholesterol ester, entrapped in the intima is thought to be oxidized by superoxide attack on apoB-100 or derivatized by attachment of aldehydic compounds to apoB-100. There is also the possibility that apoB is glycosylated. Both derivatized LDL and oxidized LDL are engulfed by foam cells which have receptors for the modified LDL. Atherosclerosis progresses through the stages of fatty streak, fibrous plaque, and complicated lesion.

The only source of cholesterol other than *de novo* biosynthesis is food. Therefore, several investigators have quantified the amount of cholesterol and its oxides in foods.

VARIATION IN QUANTIFICATION OF COPs

MANY FOOD PRODUCTS have been reported to contain some COPs. Two reviews listed such foodstuffs (Smith, 1981; Finocchiaro and Richardson, 1983). Eggs and egg-containing products have been studied extensively, essentially due to their high cholesterol content (Tsai et al., 1979; Addis, 1986; Nourooz-Zadeh and Appelqvist, 1987; Pie et al., 1990). Other foods that have been analyzed for COPs content are milk and milk products (Finocchiaro et al., 1984; Nourooz-Zadeh and Appelqvist, 1988; Sander et al., 1989a, b; Pie et al., 1990; Chan et al., 1993), meat and meat products (De Vore, 1988; Csallany et al., 1989; Nourooz-Zadeh and Appelqvist, 1989; Monahan et al., 1992a), and other processed foods including seafood (Ryan, 1982; Khayat and Schwall, 1983; Addis, 1986; Higley et al., 1986a; van de Bovenkamp et al., 1988; Oshima et al., 1993; Sarantinos et al., 1993; Osada et al., 1993b). However, results for COPs found in these foodstuffs may not be reliable due to variations in methodology such as hot vs. cold saponification and choice of analytical tools. Furthermore, intentional oxidative stress such as irradiation or extended heating may produce levels of oxidation that would not take place in food processing.

A series of cholesterol oxidation products can arise as artifacts during isolation by hot saponification (Smith, 1981). Hot alkaline saponification to hydrolyze lipid matter (including sterol esters) is commonly employed to concentrate non-saponifiable sterols before analyses. This can cause certain oxidized sterols, notably isomeric epoxides and 7-ketone to decompose to various degrees (Chicoye et al., 1968b; Tsai et al., 1980; Smith, 1981), thereby generating the diene (cholesta-3,5-dien-7-one) (Tsai and Hudson, 1984). The secondary oxidation product may have been erroneously identified in some instances as present in the original sample. Chicoye et al. (1968a, b) demonstrated that saponification at ambient temperatures could be employed to successfully extract the 7-ketone from foods containing oxidized cholesterol without generating the diene.

Eight common COPs are usually found in foods. The most common proposed cholesterol oxidation pathways in foods are presented in Fig. 3. Comprehensive cholesterol autoxidation pathways have been published by Smith (1981, 1987). Oxides include epimers of 7-hydroxycholesterol, epimers of epoxycholesterol, 7-ketocholesterol, 20-hydroxycholesterol, 25-hydroxycholesterol, and cholestanetriol. Researchers suggested that of the common COPs, cholest-3,5-dien-7-one is artifactually derived from 7-ketocholesterol, and formation of cholestanetriol results from degradation of epimeric epoxide during the analytical procedure (Smith, 1981; Park and Addis, 1985a, b). Therefore, the data in Tables 1 and 2 show only eight most commonly found COPs in various foods Fish and marine products which contain high quantities of polyunsaturated fatty acids have been surprisingly ignored (only two references were found).

Cholesterol oxides in eggs

An average egg contains 213 mg cholesterol (Anon., 1990). This is about twice the cholesterol content of butter and freeze-dried meat products and about 5-10 times more cholesterol than is found in most dairy products (Nourooz-Zadeh and Appelqvist, 1987). Fieser (1953a, b) was the first to report the finding of cholestane-3β,5α,6β-triol in powdered egg yolk, a raw material used in many convenience foods. Spray-dried and other dehydrated eggs have been widely studied for COPs since 1968 (Chicoye et al., 1968b; Tsai and Hudson, 1985; Sander et al., 1989a). Dry eggnog mix (Herian and Lee, 1985), cooked egg and dehydrated egg products (Herian and Lee, 1985; Tsai and Hudson, 1985; Nourooz-Zadeh and Appelqvist, 1987) were reported to contain COPs as well. In addition, dried mixed diets which contain dry egg powder have been a focus of study, especially in Europe where dry egg powder is widely used in regular diets (Nourooz-Zadeh and Appelqvist, 1987; van de Bovenkamp et al., 1988). Concentrations of total COPs in these products have ranged from trace amounts to 200 ppm, but COPs have not been reported in fresh eggs.

Definitive studies on isolation, identification, and quantification of cholesterol α- and β-epoxide in powdered eggs were conducted by Tsai and coworkers (1980, 1984, 1985). Briefly, the method involved chloroform-methanol extraction, silicic acid chromatography, GC and HPLC. Qualitative confirmation was accomplished by MS, NMR, and IR spectral determination. Tsai and Hudson (1984) obtained quantitative results on commercial dry egg products and found wide variation among samples. Some individual samples contained very high concentrations of epoxides. The potential for toxicity of powdered eggs may be very significant because these results did not include data on the other six common COPs or other lipid oxidation products that were probably present.

Sander et al. (1989a) found the average levels of COPs in eggs in decreasing order as follows: α-ep, 7β-OH, β-ep, and 7-keto. The α to β ratio was 2.3 to 1. Sander and his group (1989a) also found a very low level of 25 hydroxycholesterol and triol. Nourooz-Zadeh and Appelqvist (1987) found higher β-epoxides than α-epoxides with the β to α ratio equal to 5-10 to 1 depending on storage time. Also, the concentration of 7α-OH>7-keto and level of total COPs (Nourooz-Zadeh and Appelqvist, 1987) were about 5-10 times lower than that obtained by Sander et al. (1989a). Nourooz-Zadeh and Appelqvist (1987)

Table 2—Cholesterol oxides in a variety of foods[a]

Food	Treatment	Oxysterol found (mg/g) 7α	7β	ep	7-K	20-OH	25-OH	triol	others	References[b]
Egg and egg products										
dehydrated egg mix	stored for 12–18 m	0.7-1.2	0.2-1.0	α(0.3-4.0) β(2.7-3.2)	0.6-3.5	nd	nd	nt	5,6-diol (nd)	15
dehydrated egg yolk	stored for 6–12 m	2.2-8.9	2.5-9.8	α(1.3-2.5) β(6.5-12.0)	2.4-3.5	0.7-0.9	nd	nt	5,6-diol (tr)	15
dehydrated egg yolk, egg mix	stored for 8 yr (fluctuate temp and conditions)	27.5	46.8	α(9.4) β(nd)	5.7	6.6	10.4	nt	5,6-diol (27.6)	15
dried egg powder	commercially dried	nt	nt	α(nd-15) β(nd-59)	nt	nt	nt	nt	nt	28, 29
dried egg yolk powder	stored at 4°C for 1 yr or at room temp for 4 yr	nd	12.8-78	α(5.8-46) β(nt)	1.8-9.4	nd	0.3-13.9	0.0-0.5	nt	30
dried egg yolk powder	after storage, irradiated with UV light for 3 wk	nd	507	α(2522) β(nt)	200	nd	860	62	nt	30
dried egg yolk powder	commercially dried	nt	nt	α(nd-33) β(nd-133)	nt	nt	nt	nt	nt	28, 29
egg mix, 2-6% whole egg powder[x]	commercially processed, cooked	0.3-1 1.3	0.3-1.2 1.7	α(0.1-0.8) β(0.7-2.1) α(0.9) β(2.2)	0.5-1.6 2.5	0.1-0.3 0.9	tr-0.1 0.2	tr-0.1 0.2	nt	23
egg nog mix	exposed to fluorescent light for prolonged periods up to 110 days	0.0-0.8	0.0-0.5	nt	nt	nt	nt	nt	nt	7
egg powder[x]	heated at 90°C for 6–24 hr	1.6-65.2	1.2-98.1	α(0.7-18.1) β(0.4-52.2)	2.2-316.5	nt	nd-5.2	nd-1.8	nt	6
egg yolk powder	spray-dried with direct heat (outlet temp 75–150°C) with 5-300 ppm NO_x	2.4-23.4	1.7-28.5	α(1.8-20.2) β(6.9-110.0)	6.5-30.5	nt	nt	nt	nt	14
egg yolk powder[m]	commercially dried	43.2	47.4	α(32.3) β(68.5)	46.2	8.0	2.5	tr	nt	23
egg yolk powder	spray-dried with 5–10% H_2O_2, inlet temp 150–350°C, outlet temp. 90–150°C	nt	nt	nd-48	nt	nt	nt	nt	nt	13
	storage up to 7 mo	nt	nt	0-1320	nt	nt	nt	nt	nt	13
	spray-dried with antioxidant (67–200 ppm)	nt	nt	18-40	nt	nt	nt	nt	nt	13
egg yolk powder	commercial	nq	51	α(79) β(28)	22	nt	2	nq	nt	25
egg[x]	fried or boiled for 1–10 min	4-8	34-59	22-65	nd	46	nd	1-8	4β-OH (32-51) 22-keto (2-21)	27
egg powder[x]	whole egg/egg yolk	14	20-198	90-641	17-35	59-156	nd	nd	4β-OH (14-147) 22-keto (15-49)	27
fresh egg yolk		0.3	nd	α(10.7) β(nt)	0.2	nd	0.0	0.0	nt	30
omelet mix	commercial	nq	10	α(nd) β(2)	2	nt	nd	nd	nt	25
powdered egg yolk	spread as a thin layer and heated in oven at 110°C for 4 days	nt	nt	nt	nt	nt	26.4	nt	nt	10
scramble egg mix	commercially available	nt	nt	α(1-7) β(6-26)	nt	nt	nt	nt	nt	28, 29
spray-dried egg powder[x]	stored in freezer at −20°C in dark environment for 3–8 mo	1.3-3.0	1.2-1.4	α(0.8-0.9) β(2.5-2.6)	2.2-3.5	nt	nd	nd	nt	
spray-dried egg powder[x]	stored in dark glass bottle at room temp for 3–8 mo	3.9-9.2	4.4-9.2	α(1.2-3.6) β(4.5-13.6)	3.1-4	nt	nd	nd	nt	6
spray-dried egg powder[x]	stored in glass bottle, exposed to daylight for 3–8 mo	4.1-10.6	5-10.9	α(1.0-4.6) β(4.1-21.9)	3.9-4.3	nt	nd	nd	nt	6
spray-dried egg yolk	low energy UV light for 280 hr or exposed to sunlight for 5 hr	1.7	1.7	α(1.7) β(1.7)	1.7			1.7		2
spray-dried egg yolk (fresh)		7.8	8.98	α(5.26) β(4.93)	9.13	nd	nd	nd	nt	6
whole egg powder	stored at 4°C for 1 yr or at room temp for 4 yr	11.9-64.9	nt	α(4.2-35.8) β(nt)	1.9-11.2	nt	0.3-2.3	0.0-0.5	nt	30
whole egg powder[x]	commercially dried	21.6-34.4	23.0-43.3	α(16.6-47.3) β(40.3-62.7)	22.4-32.7	4.8-9.1	tr-3.0	tr	nt	23
whole egg powder	commercial	nq	nd-65	α(12-111) β(5-46)	nd-37	nt	nd-10	nd-6	nt	25

—Continued on page 1164

Table 2—Cholesterol oxides in a variety of foods[a]

Food	Treatment	Oxysterol found (mg/g) 7α	7β	ep	7-K	20-OH	25-OH	triol	others	References[b]
Dairy products										
butter[x]	heated at 170–180°C for 10–20 min and stored at −20°C for 3–6 mo	1.6-8.9	1.9-14.9	α(1.0-7.4) β(4.4-18.4)	5.0-14.4	tr-0.6	tr-0.6	tr-0.4	nt	23
butter[x]	unheated and stored for 3–6 mo	nd-0.22	nd	α(nd) β(nd-1.52)	0.42-0.97	nd	nd	nd	nt	23
butter, fresh[x]	heated at 170–180°C for 10–20 min	1.2-3.9	1.7-4.6	α(1-2.9) β(4.3-7.3)	5.1-8.6	tr	tr	tr	nt	23
butter oil	bleached with benzoyl peroxide stored at 15°C for 90 days and −20°C for 1 yr under air or N_2	10-60	20-90	nd-30	nt	nt	nt	nd	nt	5
butter oil (salted/unsalted)	during prolonged heating at 110°C	1-130	1-90	α(1-230) β (1-200)	1-350	nt	nd	37	nt	26
	various salt (NaCl) concentration, stored at 0–16 days	nd-109	14-111	α(13-191) β(9-193)	14-308	nt	nd-6	2-22	nt	26
butter powder	stored at 4°C for 18 mo	nd-5	nd-8	α(nd-42) β(nd-9)	nd-6	nt	nd-7	nd-9	nt	26
cheese blue	dehydrated	nd	nd	α(nd-3) β(nd)	nd-4	nt	nd	nd	nt	25
Cheddar	dehydrated	nd-6	nd-9	α(nd-9) β(nd-4)	nd-14	nt	nd-3	nd-17	nt	25
Parmesan	dehydrated	nd	nd-9	α(nd-5) β(nd-6)	nd-16	nt	nd-4	nd-9	nt	25
Romano	dehydrated	nd	nd-2	α(nd-5) β(nd)	nd	nt	nd	nd	nt	25
cheese Cheddar	12 wk of exposure to fluorescent light	nd	2-6	α(2-20) β(1-4)	nd-8	nt	nd	nd	nt	26
cheese powder Cheddar, blue, Parmesan, Romano	stored at 21°C and 38°C for 6 mo	nd	nd-11	α(nd-10) β(nd-3)	nd-12	nt	nd-6	nd-3	nt	26
	stored at 4°C for 18 mo	nd-6	nd-22	α(nd-16) β(nd-13)	nd-39	nt	nd-4	nd-17	nt	26
cheese Parmesan, Romano	commercially available, grated	nq-6	nq-6	6-32	nt	nt	nt	nq-2		5
dried full-fat milk	after 2–7 yr storage	2.9-3.9	nt	α(1.2-4.1) β(nt)	0.5-1.5	nt	0.1-0.8	<0.1-0.3	nt	30
fresh milk product sour cream, milk fat, butter, three cheese spread, cottage cheese, evaporated milk, cream cheese, vanilla ice cream, vanilla yogurt and two whole milk		nd-2	nd-7	α(nd-12) β(nd-3)	nd-4	nt	nd	nd-1	nt	25
Indian ghee[x] (cow, buffalo)	laboratory prepared	2.1-3.6	4.2-5.9	4.6-5.6	3.1-4.1	nd-2.7	nd	nd	nt	11
Indian ghee[x] (cow, buffalo)	fried at 180–200°C for 1 hr repeatly 3 times over 3-day period	7.1-33.8	22.1-86.9	23.8-95.6	14.0-84.6	tr-8.3	3.1-16.2	tr-7.3		11
Indian ghee[x] (cow, buffalo)	intermittent heating at 225°C for 30 min, stored 24 hr, repeated for 3 days	2.9-22.9	7.8-45.9	13.1-68.8	7.9-47.7	tr-7.7	2.2-10.8	tr-7.6	nt	11
Indian ghee[x] (cow, buffalo)	control over 3-day period	3.1-5.2	5.5-12.8	7.5-12.4	3.3-6.5	nd-1.6	nd	nd	nt	11
infant formula powder	dehydrated	nd-8	6-9	α(nd-3) β(nd-46)	11-18	nt	nd-19	nd-35	nt	25
milk powder	direct/indirect heating with different packages	0.48-2.88	0.96-2.4	α(0.12-1.44) β(1.2-7.2)	1.92-18.72	nt	nt	nt	nt	1
	with oxygen absorber	nd	nd	nd	0.07-0.14	nt	nt	nt	nt	1
milk products (sour cream, butter powder, NFDM, choc. powder, malted milk powder)	dehydrated food	nd-4	nd-3	α(nd-26) β(nd-3)	nd-7	nt	nd	nd-3	nt	25
powder full cream milk	spray-dried	2-14	2-3	2-8	5	6-11	nd	1	4β-OH (nd) 22-keto (3-10)	27
powdered milk skim[m]	high heat spray-dried, stored for 13 hr in a warehouse	0.05	0.07	α(0.02) β(0.007)	0.05	0.007	0.003	nd	nd	16
skim milk powder	stored from 13–37 hr	0.04-0.16	0.07-0.23	α(0.009-0.04) β(0.02-0.09)	0.05-0.19	0.006-0.03	0.003-0.008	nd	5β,6β-diol (nd-0.02)	16
skim milk powder	high heat spray-dried	0.01-0.02	0.02	α(0.008-0.03) β(0.01)	tr-0.025	nd	nd	nd	nd	16

—Continued on page 1165

Table 2—Cholesterol oxides in a variety of foods[a]

Food	Treatment	Oxysterol found (mg/g) 7α	7β	ep	7-K	20-OH	25-OH	triol	others	References[b]
whole milk	high heat spray-dried (not detected in low and medium heat treated)	nd-0.34	nd-0.14	α(0.14) β(0.05-0.07)	tr-0.1	nd	nd	nd	nd	16
whole milk powder	12 hr old	7.2×10^{-2} -0.24	0.16-0.36	α(0.12-0.6) β(0.26-0.77)	1.22-2.21	nd	nd	nd	5β,6β-diol (tr-7.2×10^{-2})	16
sour cream powder	stored at 4°C for 18 hr	nd	nd-7	α(nd-14) β(nd-5)	nd-6	nt	nd	nd	nt	26
Meat and meat products										
bacon, fried (not found in raw bacon and bacon drip)	fried at 170–200°C for 10 and 20 min	nd-0.3	nd-0.3	α(nd-0.2) β(nt)	nd-0.2	nd	nd-0.5	nd	nd	17
beef, pork, veal[x]	pan-cooking of minced meat for 3–10 min	0.51-0.64	0.38-0.85	α(0.33-0.55) β(0.84-1.31)	1.66-2.25	0.1-0.3	0.19-0.38	nd-0.07	nt	24
beef, pork, veal[x]	unirradiated (stored at 4°C for 0–2 wk)	nt	nt	α(0.02-0.18) β(0.03-0.6)	0.03-1.52	nt	nt	nt	4-cholestene-3-one (0.03-0.46) 4,6-cholest-diene-3-one (0.02-0.08) 4-cholestene-3,6-dione (tr-0.07)	9
beef, pork, veal[x]	irradiated (stored at 4°C for 0–2 wk)	nt	nt	α(0.04-0.58) β(0.09-1.66)	0.12-4.43	nt	nt	nt	4-cholestene-3-one (0.07-0.48) 4,6-cholest-di-ene-3-one (0.02-0.08) 4-cholestene-3,6-dione (tr-0.13)	9
beef tallow	heated at various frying temp for up to 216 hr	nt	nt	α(10-42.8)	9.1-43.7	nt	nt	nt	nt	21
chicken (7 samples) beef (7 samples) turkey (2 samples)	freeze dried	nd-17	nd-18	α(nd-43) β(nd-27)	nd-27	nt	nd	nd-2	nt	25
freeze dried pork[m]	stored for 3 yr at room temp	90.9	68.4	α(12.5) β(nq)	259.8	nt	nd	28.4	nt	22
freeze-dried pork[m]	stored for 3 yr in presence of light and air	28.98	21.05	nt	126.59	nt	nd	nt	nt	3
ground beef	-raw	nt	nt	nt	nd-0.6	nt	nt	nt	nt	4
	-cooked (1 min in microwave) store at 4°C for 4 days	nt	nt	nt	nd-6.2	nt	nt	nt	nt	
ground pork fed with α-tocopherol	cooked in retortable bag at 70°C 30 min; store @ 4°C in refrig. 2–4 days		2.6-5.4	α(nt) β(3.2-7.2)	4.0-10.9	nt	nt	nt	nt	12
lard	spiked with 2 and 10 times cholesterol level, heated at 180°C for 16–25 days	0-100	0-150	α(0-65)	0-160	nt	nt	0-20	nt	31
lard	-chemically refined stored at 50°C up to 18 days	tr	nd	α(nq-0.5) β(nt)	nd-0.2	nd	nd	nt	nt	17
	-consumer package in cold storage (2–18 mo)	tr-0.3	nd	α(0.2-0.4) β(nt)	tr-0.3	0.2-0.5	tr-0.2	nt	nt	17
	-refined, unrefined	nd-tr	nd	α(nd-0.3) β(nt)	tr-0.3	nd-0.3	nd-0.2	nt	nt	17
meat products	raw or cooked	tr-1640	tr-34	nt	tr	tr	tr	tr-1335	tr-1869	8
pork muscle normal or Porcine-stress syndrome	fresh	nd	nd	nt	nd	nt	nd	nt	nt	3
	normal, unwrapped, refrigerated at 40°F for 10 days	nd	nd	nt	1.13	nt	nd	nt	nt	
pork muscle (fresh)	irradiated with UV light to 72 hr	nd-2.01	nd-2.29	nt	nd-2.26	nt	nd	nt	nt	3

—Continued on page 1166

Table 2—Cholesterol oxides in a variety of foods[a]

Food	Treatment	Oxysterol found (mg/g) 7α	7β	ep	7-K	20-OH	25-OH	triol	others	References[b]
raw beef[x]	stored at 0–4°C for 7 to 21 days	nt	nt	α(0.13-0.37) β(0.44-0.76)	1.02-2.55	nt	nt	nt	nt	33
raw beef, pork, veal[x]		0.18-0.33	0.21-0.34	α(0.17-0.42) β(0.35-1.06)	0.71-1.12	nd-0.18	0.05-0.14	nd-0.04	nt	24
raw beef, pork, chicken and veal[x]		nt	nt	α(0.02-0.13) β(0.04-0.37)	0.06-0.83	nt	nt	nt	nt	33
Marine products										
Alaskan Pollack roe	pickled and spiced	38	58	α(8) β(10)	33	nt	nt	3	nt	18
anchovy, shrimp	boil-dried	nt	3.7-55.8	α(tr-18) β(tr-43.3)	4.0-60.6	nt	0.6-8.5	tr-39.1	nt	19
marine product (anchovy, Northern cod, Pacific cod, Japanese whiting, Pacific saury, Pacific herring)	salt-dried	nt	2.9-37.1	α(tr-13.1) β(tr-33.3)	2.3-48.8	nt	tr-10.7	tr-5.3	nt	19
salmon	smoked	nt	7.3	α(2.4) β(3.3)	6.3	nt	4.8	2.7	nt	19
seafood (sardine, squid, Alaskan Pollack roe)	fresh	nd	nd	nd	nd	nt	nt	nd	nd	18
seafood (sardine, squid)	dried, canned	0-27	28-98	α(2-14) β(7-49)	19-53	nt	nt	nd	unknowns (36-73)	18
Miscellaneous processed products										
athletic supplement protein powder	dehydrated	4	2	α(nd) β(20)	20	nt	24	16	nt	25
baby food	canned	nd	nd-11	α(2-5) β(1-3)	1-6	nt	nd-2	nd-2	nt	25
baby food[x] egg and steak, egg custard, caramel custard	commercially available	nd	1	1	1	1-2	nd	nd	4β-OH (1) 22-keto (1)	27
baby food containing beef, turkey, chicken	containing dehydrated meat	nd	nd-24	α(nd-4) β(nd-5)	5-7	nt	9-14	4-5	nt	25
cake mix	commercial	1-2	1-3	1-4	nd-5	2-15	nd-1	nd-2	4β-OH (nd-6) 22-keto (1-10)	27
cake mix (with butter powder and egg powder) in[x]	commercial	0.29-0.36	0.08-0.43	α(0.22-0.45) β(0.9-1.45)	1.6-1.7	tr-0.17	tr-0.12	tr-0.08	nt	23
commercial cookies[x]	stored for 1–12 mo	0.1-0.6	0.21-0.68	α(0.22-0.51) β(0.41-1.7)	0.64-1.29	nt	nd	nd	nt	6
cookie mix[x]	commercial	0.16-0.18	0.12-0.14	α(0.1-0.2) β(0.5-0.7)	0.68-0.75	tr	tr	tr	nt	23
croissant[m]	commercial	0.07	0.07	α(0.14) β(0.25)	0.46	tr	0.05	0.06	nt	23
duplicate dutch diet	raw, fried, baked, or grilled plus extra fruit and vegetables	1.7-2.3	nt	α(0.7-1.7) β(nt)	1.0-2.0	nt	0-0.2	<0.1-0.1	nt	30
French fries	purchased from different restaurants	2	2-3	α(4-17) β(3-4)	3-5	nt	nd	nd	nt	32
French fries	deep fat fried in animal/vegetable shortening (follow for 30 days)	nd	1-3	α(nd-3) β(nd-2)	4-18	nt	2-7	3-16	nt	32
French fries	fried in tallow	nd	6.8-53.8	nt	4.1	nt	nt	nt	nt	20
frozen dinner Quiche Lorraine cheese canelloni	raw/cooked	nd-1	4-13	nd-4	nd-4		nd	nd-8	4β-OH (nd-1) 22-keto (nd-47)	27
health food raw cow's brain and raw liver prep[x]	tablet form	nd-46.1	nd-37.6	nt	13.8-70.1	nt	nt	nt	nt	20
mayonnaise		5	nd	nd	13	7	9	2	4β-OH(32) 22-keto (nd)	27
pancake mix		nd	nd	nt	1.1	nt	nt	nt	nt	20
sweet biscuit	commercial	1-8	7-23	2-5	1-13	1-46	nd-2	1-3	4β-OH (1-18) 22-keto (1-2)	27

—Footnote at top of page 1167

[a] nt = not tested, nd = not detected, nq = detected but not quantified; tr = trace;
[b] Ref. 1-Chan et al (1993); 2-Chicoye et al. (1986b); 3-Csallany et al. (1989); 4-De Vore (1988); 5-Finocchiaro et al. (1984); 6-Fontana et al. (1993); 7-Herian and Lee (1985); 8-Higley et al. (1986a); 9-Hwang and Maerker (1993); 10-Kou and Holmes (1985); 11-Kumar and Singhal (1992); 12-Monahan et al. (1992a); 13-Morgan and Armstrong (1987); 14-Morgan and Armstrong (1992); 15-Nourooz-Zadeh and Appelqvist (1987); 16-Nourooz-Zadeh and Appelqvist (1988); 17-Nourooz-Zadeh and Appelqvist (1989); 18-Osada et al. (1993b); 19-Oshima et al. (1993); 20-Park and Addis (1985a); 21-Park and Addis (1986b); 22-Park and Addis (1987); 23-Pie et al. (1990); 24-Pie et al. (1991); 25-Sander et al. (1989a); 26-Sander et al. (1989b); 27-Sarantinos et al. (1993); 28-Tsai and Hudson (1984); 29-Tsai and Hudson (1985); 30-van de Bovenkamp et al. (1988); 31-Yan and White (1990); 32-Zhang et al. (1991); 33-Zubillaga and Maerker (1991)
[c] = range of means were tabulated;
[d] = mean values were tabulated.

found quantifiable levels of 25-hydroxycholesterol and cholestanetriol but only after 8 yr of storage. Tsai and Hudson (1984) used HPLC for determining the epoxide level in spray dried whole egg and yolk products and found the β to α ratio was 2.7 to 1.

Morgan and Armstrong (1987) demonstrated that the presence of pro-oxidants (e.g., hydrogen peroxide) during spray-drying was necessary to promote formation of measurable quantities of cholest-5,6-epoxides in egg yolk powder dried with an indirect-heat system. Egg yolks dried at 150°C outlet temperature in the presence of 300 ppm NO_x contained total epoxide levels that approached the value generated by H_2O_2 (Morgan and Armstrong, 1992).

Fontana et al. (1993) used ^{1}H-NMR to quantify the COPs in spray-dried egg powder. 25-Hydroxycholesterol and cholestanetriol were found only in the egg powder that had been heated at 90°C for 6 to 24 hr (Fontana et al., 1993). Heating the egg sample also caused a sharp increase in 7-ketocholesterol (from 2.2 ppm to 317 ppm).

Cholesterol oxides in dairy products

Several dairy products and milk powder are reported to contain oxidized cholesterol (Nourooz-Zadeh and Appelqvist, 1988; Sander et al., 1989a, b; Pie et al., 1990). Nourooz-Zadeh and Appelqvist (1988) noted that the quantitative pattern for concentration of the 8 COPs in milk powder products was fairly similar to that observed in analysis of dehydrated egg yolk and its mix products (Nourooz-Zadeh and Appelqvist, 1987). However, the 7-ketocholesterol was in relatively higher concentration in milk powder products than in egg yolk powder (Nourooz-Zadeh and Appelqvist, 1988). It is highly likely that differences in mineral composition of the products govern the proportion of 7-ketocholesterol to 7-hydroxycholesterol (Smith, 1981). Results also showed that fresh milk and milk products contained none or only trace amounts of COPs (Finocchiaro et al., 1984; Nourooz-Zadeh and Appelqvist, 1988; Sander et al., 1989a). Of all the fresh milk products Sander et al. (1989a) tested, only vanilla yogurt contained as many as six out of seven COPs (Table 2). Other products have rarely contained COPs other than α-ep (Sander et al., 1989a). Samples of cheese spread, cottage cheese, evaporated milk, and whole milk did not contain any of the targeted COPs.

Finocchiaro et al. (1984) applied TLC to analyze butter oil and grated cheese for COPs. Positive samples were analyzed by HPLC and MS. Both bleached butter oils and grated aged cheese displayed trace levels of COPs . However, the data were questionable because not all samples were confirmed by MS.

Cholesterol oxides in meat and meat products

The mean lipid content of lean meat is 10%, wet weight basis, of which triglycerides and phospholipids are major components and cholesterol is a lesser component, ranging from 50 to 89 mg% (Rhee et al., 1982; Pikul et al., 1984). Beef tallow was the first sample tested for COPs in meat and meat products (Ryan et al., 1981), but no quantifications were made. The oxidation of cholesterol during heating of tallow, as revealed by TLC, was reported by Ryan et al. (1981). Park and Addis (1985a, 1987) did not detect 7-ketocholesterol in raw beef and beef products, but found the compound in 3-yr-old freeze-dried pork and broiled beef steak and turkey. Sander et al. (1989a) reported ppm quantities of 7-ketocholesterol and the 2 epoxides in dehydrated chicken, beef, and turkey. Zubillaga and Maerker (1991) analyzed the irradiated meat for 7-ketocholesterol and the epoxides and found that all 3 cholesterol oxidation products increased over 3-wk storage. 7-Ketocholesterol was the principal product and constituted well over 50% of the mixture (Zubillaga and Maerker, 1991). While the recovery of 7-ketocholesterol and α-epoxide was ≈97%, the recovery of β-epoxide was only 62%. The researchers suggested that hydration of both epoxides resulted in formation of the triol. In aqueous acidic media the β-epoxide is converted to the triol much more readily than the α-epoxide (Maerker, 1987; Lakritz and Maerker, 1989). This was confirmed by examination of the fraction 2 eluate from meat to which the β-epoxide had been added. Results showed a substantial amount of triol (Zubillaga and Maerker, 1991), which, however, was not quantified. Triol was apparently absent in eluates from meat to which epoxides had not been added. They indicated that cholesterol epoxides normally found in meat were protected from contact with the acidic aqueous medium by the hydrophobic property of the cell membrane.

Researchers have found that similar to other fresh foods from animal origin, fresh meat and meat products contain none or only trace amounts of COPs (Park and Addis, 1987; Pie et al., 1991). In contrast to other studies, the technique used by Hwang and Maerker (1993) were more sensitive and they detected some COPs in fresh meat (beef, pork, and veal). Among the compounds, 7-ketocholesterol and β-epoxide were dominant COPs in each type of fresh meat.

An unusual amount of COPs in meat products was reported by Higley et al. (1986a). A cooked bratwurst contained large amounts of the B-ring COPs, namely, 7β-hydroxycholesterol (1640 ppm) and cholestanetriol (1335 ppm). It also contained the side-chain COP, 22-ketocholesterol (1869 ppm) (Higley et al. 1986a). Higley et al. (1986a) also reported high levels of COPs in other processed meats including raw hamburger, cooked lean bacon, and beef franks.

Higley et al. (1986a) used TLC as a screening tool, then randomly selected 10 of 26 meat samples for further purification and HPLC. Their data presented the measurable amounts of COPs in the meat samples analyzed by HPLC. Identification of particular oxides was based on co-elution with known standards. Their analyses indicated unusu-

ally high concentrations of triol and 22-ketocholesterol and are questionable. Higley et al. (1986a) admitted that they may have overestimated COPs due to multiple compounds co-eluting with the two oxides. They observed that the two oxides elute close to the solvent front where the unidentified non-polar components eluted despite the multiple steps applied. As discussed for the case of triol, the chromatogram revealed several compounds eluting at the solvent front. They suggested that these results could be attributed to non-cholesterol compounds that absorb at the 240 nm absorption maximum. They concluded that HPLC detection was not specific for reliable analysis. Furthermore, the duplicate samples for triol determinations of cooked bratwurst and raw hamburger did not agree. One showed unexpectedly high content (more than 1200 ppm) while the other showed no triol. The same results were reported when duplicate samples were analyzed for other oxides. In many meat samples, trace levels of individual COPs were found in only one of duplicate analyses. The lengthy extraction procedure used to obtain HPLC-analyzable sample had limitations in time and yield. Nevertheless, they concluded that the concentrations of certain COPs in meats were minimal. While HPLC analyses of chicken roll, raw or cooked hamburger, and cooked bratwurst samples suggest the presence of several COPs, the concentrations are below the limits of quantification. In addition, the concentrations of the COPs were below concentrations to provide toxic responses in cell culture (Higley and Taylor, 1984).

Table 3—Unusua cholesterol oxides in some muscle foods[a]

	Oxysterol found (μg/g)									
	19-OH	22-keto	20-OH	22-OH	25-OH	6-keto	7-keto	7β-OH	7α-OH	triol
chicken roll	tr	—	tr	tr	tr	18	tr	tr	tr	tr
cooked burger	tr	—	—	tr	tr	tr	tr	—	72	—
cooked bacon	1640	668	—	—	tr	—	tr	—	tr	tr
cooked bratwurst	—	1869	tr	—	tr	tr	—	tr	1640	1335
turkey bologna	—	tr	—	—	—	tr	—	—	—	86
slice beef loaf	—	34	—	—	—	—	—	—	—	tr
raw burger	—	210	—	tr	—	tr	tr	3.6	tr	1298
salt pork	—	247	—	—	—	—	—	—	—	—
bratwurst	—	—	—	tr	—	tr	tr	tr	—	—
beef franks	—	—	—	—	tr	—	—	34	56,85	tr

[a] Modified from Higley et al., 1986a. tr = trace, — = not detected

Cholesterol oxides in marine products

Two researchers have described the occurrence of COPs in marine products (Oshima et al., 1993; Osada et al., 1993b). They concentrated on traditionally processed seafoods which are consumed extensively in Japan. Oshima et al. (1993) analyzed cholesterol and its oxides in dried fish products. Moisture contents of the fish products were relatively low, 10.1 to 28.9%. The level of total COPs ranged widely between 8.3 ppm in boiled and dried shrimp to 188 ppm in boiled and dried anchovy. Of all fish products tested, anchovy seemed to have the highest COPs among the marine products.

The B-ring COPs were dominant in fish products although oxidation in the side-chain of cholesterol yields the isomers of 20-, 24-, 25-, and 26-hydroxycholesterol (Korahani et al., 1982; Maerker and Unruh Jr., 1986). Only one side-chain COP (25-hydroxycholesterol) was found in the fish products examined (Oshima et al., 1993). However, the presence of other COPs cannot be excluded. A typical gas chromatogram of the salted-dried anchovy revealed several unidentified peaks and, therefore, side-chain COPs other than 25-hydroxycholesterol probably were present.

Among the COPs detected in marine food products by Osada et al. (1993b), 7α-,7β-hydroxycholesterol, β-epoxide, α-epoxide, cholestanetriol, and 7-ketocholesterol were identified. A relatively large amount of 7β-hydroxycholesterol occurred in all processed marine foods. Osada et al. (1993b) also analyzed the fatty acid composition to show the correlation between degree of unsaturation and cholesterol oxidation. All samples contained abundant amounts of 20:5 n-3 (eicosapentaenoic acid) and 22:6 n-6 docosahexenoic acid. Squid and pollock roe contained 22:6 n-6 at a relatively high proportion.

Oshima et al. (1993) and Osada et al. (1993a) also examined the rate of cholesterol oxidation in the presence of other lipids by model systems. The model systems of Oshima et al. (1993) consisted of a mixture of purified cod liver triglycerides plus cholesterol, a mixture of authentic triolein plus cholesterol, triolein alone, and cholesterol alone. All systems were stored separately at 25°C in dry air for up to 104 days. Osada et al. (1993a) heated cholesterol with or without various fats (tristearin, beef tallow, triolein, soybean oil, safflower oil, linseed oil, and sardine oil) at 100°C for up to 24 hr. Both model systems showed that cholesterol was stable, and essentially no oxidized cholesterol was produced when heated alone. However, when fats were present, cholesterol was unstable, in particular when heated with unsaturated fats. The observations indicated that peroxidation of unsaturated fatty acids precedes cholesterol oxidation. It is presumed that radicals produced from PUFAs acted as accelerators of cholesterol oxidation, because cholesterol itself is oxidized in a radical chain oxidation system (Korahani et al., 1982). These results strongly suggest that cholesterol oxidation in fish products proceeds in conjunction with oxidative decomposition of the coexisting polyunsaturated fatty acids of fish oils (Oshima et al., 1993; Osada et al., 1993b).

Cholesterol oxides in other processed foods

Zhang et al. (1991) found 9 ± 3 ppm triol and 20-24 ± 6-9 ppm total COPs in French fried potatoes. They suggested that French fried potatoes and other deep-fried foods cooked in animal/vegetable fat were a major source of COPs in the U.S. diet. The conclusion is questionable. Even if it were true, tallow is no longer widely used. The amount of COPs in the diets including fried foods would depend on several factors such as eating habits of each individual, the kind of frying medium, the serving size, and frequency of consumption.

According to van de Bovenkamp et al. (1988), in duplicates of the average diet eaten in the Netherlands, oxysterol ranged from 0.0-0.2 μg/g for 25-hydroxycholesterol to about 2 μg/g for 7β-hydroxycholesterol. The concentrations were not higher in duplicate diets made of fried, baked, and grilled products than in duplicate diets made of equivalent raw foods. Thus, the amount of cholesterol oxidized during frying, baking or grilling of foods appears to be very small. As the daily food intake for an average person in the Netherlands is about 500g, the average consumer there would ingest about 1 mg 7β-hydroxycholesterol and 0.5 mg cholesterol α-epoxide per day. The diets to which fruits and vegetables of presumably an-

ticarcinogenic activity had been added, contained somewhat higher levels of 7-ketocholesterol (2.0 μg/g compared with 1.1 and 1.0 μg/g for the other two mixed diets without the extra fruits and vegetables).

These values may be considered upper limits because of freeze-drying and pelleting that the samples had undergone. However, the chromatogram of these duplicate diets contained several peaks that were not identified. Therefore, normal Dutch food might contain appreciable amounts of oxysterols other than those identified. The oxysterols studied by van de Bovenkamp et al. (1988) are those generally formed from cholesterol under a range of conditions. Therefore, van de Bovenkamp et al. (1988) suggested that the most plausible load of oxysterol in the average Dutch diet was, at most, a few mg/day. Whether such amounts contribute to the development of chronic disease is not known.

One interesting trend was noted among the variety of foods examined. Two cake mixes and one sweet biscuit mix contained the full complement of COPs. All of the COPs probably occurred due to the combination of eggs and dairy products in such foods.

FACTORS THAT AFFECT CHOLESTEROL OXIDATION

MANY FOODS have been analyzed because of their cholesterol content and exposure to conditions known to promote oxidation of cholesterol. Those conditions include application of heat, exposure to light and prolonged storage. The degree of oxide formation is related to processing temperature, length of heating time, storage conditions, level of activator present, and packaging (Table 2). The effect of ionizing radiation on cholesterol has also been examined in various model systems (Ansari and Smith, 1979; Sevanian and McLeod, 1987; Lakritz and Maerker, 1989; Maerker and Jones, 1991). Finocchiaro and Richardson (1983) identified 8 common COPs in certain cholesterol-rich foods subjected to oxidative conditions such as elevated temperatures, prolonged storage or processing in the presence of pro-oxidants. The factors studied have included processing or heating, storage and packaging, and γ-irradiation. In addition, suggestions for prevention of cholesterol oxidation have been made by some.

Effect of processing/heating

Overall results from several sources showed that fresh foods contained none, or undetectable levels of COPs. Most of the oxides found were in foods subjected to processing conditions or exposure to heat. Kou and Holmes (1985) showed that fresh egg yolk powder contained no 25-hydroxycholesterol, but after heating at 110°C for 4 days the level rose to 26.4 ppm. Park and Addis (1986b) showed that the level of 7-ketocholesterol increased linearly with heating time but not with temperature. Kumar and Singhal (1992) showed that the COPs content of ghee increased sharply when subjected to an intermittent cooking and cooling cycle. Cooking of foods under standard domestic conditions increased production of COPs (Finocchiaro et al., 1984; Hubbard et al., 1989; Kumar and Singhal, 1991; Morgan and Armstrong, 1992).

Sarantinos et al. (1993) analyzed many foods commonly consumed in Australia. COPs were not detected in fresh egg yolk (Sarantinos et al., 1993). This was in accordance with data reported by other scientists (Chicoye et al., 1968b; Kou and Holmes, 1985; Nourooz-Zadeh and Appelqvist, 1987). Sarantinos et al. (1993) reported that prolonging cooking time increased the COPs content of fried and boiled eggs. An increase in production of COPs was related to cooking time in lyophilized (phospholipid-free) egg yolk. In addition to the 8 common COPs, they also determined the 4β-hydroxycholesterol, 22-ketocholesterol, and 20α-hydroxycholesterol. The level was 0.14–1.5 ppm, 0.02–0.49 ppm, 0.46–1.6 ppm, respectively, for the three compounds in cooked eggs and dry egg powder. No 4β-hydroxycholesterol was found in milk powder, while the 22-ketocholesterol was 0.03-0.1 ppm and 20α-hydroxycholesterol was 0.06–0.11 ppm (Sarantinos et al., 1993). Total COPs were usually $< 5\%$ of total cholesterol in processed or heated egg or milk products. However, in sweet biscuit containing eggs and milk powder the oxide contents were 20–45.7% of total cholesterol as compared to 16–29% and 37.2% in cake mix and mayonnaise, respectively. Cooking also increased the concentration of COPs in frozen dinners. Again, the loss of cholesterol did not correspond to the increase in oxides.

Tsai and Hudson (1985) reported up to 166 ppm total epoxides in commercial egg yolk powders dried with direct heating compared to 5 ppm in those dried with indirect heating. Tsai and Hudson (1985) suggested that exposure to nitrogen oxides (by-products of combustion) may be responsible for the higher levels. Nitrogen dioxide initiates autoxidation of fatty acids (Pryor and Lightsey, 1981). Tuohy and Kelly (1989) found that thiobarbituric acid values (TBA) were higher in spray-dried milk powders processed by direct firing with liquid petroleum gas or natural gas than in powders manufactured with indirect heating. Also spray-dryer outlet air temperature has been shown to be the only operating parameter that affected formation of cholest-5,6-epoxides (Morgan and Armstrong, 1987, 1992). However, the outlet temperature during commercial drying of eggs is typically 50-80°C. Although data on NO_x level are not available, such high NO_x levels (300 ppm) would not be expected (Morgan and Armstrong, 1992).

Sarantinos et al. (1993) also compared the ratios of specific oxysterols to cholesterol, before and after cooking. Primary oxysterols showed the largest increase, whether they were oxidized on carbon 7 or on the side chain. However, the side chain oxidized ones represented only a small portion of the total oxysterol. The secondary oxysterols (cholesterol epoxides and cholestanetriol) showed little or no increase, which was probably due to the short heating period.

Effects of oven cooking on COPs of meat (beef, veal, and pork) were evaluated by others (Pie et al., 1991). It was reported that total COPs increased from 69% to 487% for different oxides in some samples with pork having the greatest increase. The effect from oven cooking seemed to be more pronounced than for pan frying. Storage at −20°C for 3 mo was studied but no details were reported. It was reported that total COPs increased after storage and C-7 oxidized products were predominant with 7-ketocholesterol the most abundant (Pie et al., 1991).

Park and Addis (1986b) reported that cholesterol was oxidized to isomeric 7-hydroxycholesterols, 7-ketocholesterol and epimeric epoxides in tallow heated at 155°C for 376 hr. 7-Ketocholesterol was produced without formation of epimeric 7-hydroxycholesterol as a decomposition product of 7-hydroperoxides because 7-hydroxycholesterol was readily dehydrated in the absence of water at elevated temperatures, such as 155°C.

Sander et al. (1989a) found that canned baby food contained much

smaller quantities of COPs than dehydrated baby food which was also true for milk products (dehydrated more than fresh). The foods containing highest levels of COPs were dehydrated egg and dehydrated meat products (Sander et al., 1989a).

Park and Addis (1987) studied rare and well done beef steaks. 7β-Hydroxycholesterol and 7-ketocholesterol were suspected to be present, but concentrations were low. Therefore, precise quantification and further MS analysis were not attempted. When precooked beef products were analyzed during 12 days storage at 4°C, no detectable amounts of COPs were found. Interestingly, TBA analyses of this precooked beef product suggested that lipid oxidation development was not noticeable throughout storage.

Thermal decomposition of C-7 hydroperoxides has been reported to give rise to stable autoxidation products, like 7α-hydroxy and 7β-hydroxycholesterol and the dehydration product, 7-ketocholesterol (Smith, 1987). A preference for the 7β-epimer over the 7α-epimer, possibly due to greater thermodynamic stability of the equatorial (7β) over the axial (7α) epimer, has been reported (Kimura et al., 1979). Park and Addis (1985a) analyzed for C-7 COPs by HPLC in fried chicken meat and cooked hamburger from fast-food restaurants. In such products they did not find detectable levels of C-7 COPs, even though 30-70 ppm were detected in dried brain. In another study (Park and Addis, 1985b), the investigators used GC with a capillary column to detect some indication of cholesterol oxidation in cooked beef, but levels were too low to permit quantification or confirmation. Results indicated that beef, whether freshly cooked or precooked and refrigerated for several days, represents a very minor contributor of COPs in the diet of humans. COPs in cooked turkey samples were low but somewhat higher than beef. Further studies are needed on pre-cooked turkey and comprehensive investigations should be performed on products which contain cholesterol and are dried and stored for extended times.

Studies on the effects of heating were mostly conducted in frying oil such as tallow and lard. Park and Addis (1985a) developed a HPLC method for detection of 7-ketocholesterol. They found formation of 7-ketocholesterol was nearly linear with heating time, reaching about 10% of the initial cholesterol content at 376 hr heating at 155° C (Park and Addis, 1985a). After 376 hr, the 7-ketocholesterol began to degrade and levels of COPs began to decline (Park and Addis, 1985a). Park and Addis (1986b) noted that cholesterol loss ceased in 2 heated tallow samples when 40-45% of the initial cholesterol was gone. This suggests a distribution of cholesterols into various phases such as micelles where cholesterol would not be accessible (Park and Addis, 1986a, b).

Ryan et al. (1981) used TLC to demonstrate development of COPs in tallow heated to frying temperatures. Park and Addis (1985a) used HPLC to analyze COPs in French fries cooked in tallow and noted the existence of C-7 COPs. But because of the detection limitation of the HPLC method, other studies by Park and Addis (1986a, b) applied GC for quantification of COPs in tallow heated to the temperature commonly used in fast-food restaurants. All positive chromatographic findings were confirmed by GC-MS. Heating tallow at 155°C or 190° C resulted in loss of half the initial content of cholesterol at 250 hr, and samples heated at 190°C were affected slightly more than those at 155°C. After 250 hr at 155°C, 7% of the cholesterol had been converted to 7-ketocholesterol with lesser quantities of 7α- and 7β-hydroxy (1.5 and 2.5 %, respectively) and α-epoxide (4%) derivatives of cholesterol (Park and Addis, 1985a), with only 50% of the original cholesterol remaining. A subsequent study confirmed and extended these findings by demonstrating formation of 1.2 and 1.1% of the cholesterol into 7-keto and α-epoxide derivatives, respectively, after 70 hr at 135°C. Cholesterol degradation was also delayed by α-tocopherol (100 ppm) plus ascorbyl palmitate (500 ppm) (Park and Addis, 1986a).

Lard with two levels of cholesterol added was studied by Yan and White (1990). The rates of cholesterol loss for lard with 10 times the normal cholesterol level heated at 180° C for 10 hr/day for 24 days were compared with lard with 2 times the cholesterol level heated at 180°C for 10 hr/day for 16 days. Rates of reaction, k, were -1.18×10^{-3} hr^{-1} and -9.45×10^{-3} hr^{-1}, respectively (Yan and White, 1990). Accumulation of COPs occurred during both heating tests. Consistent with other research, the amount of COPs formed did not equal the cholesterol loss. The reason for this may be thermal degradation. During heating, thermal degradation of cholesterol likely occurred, but degradation products were not detected or identified.

Several studies followed COPs in pork resulting from processing conditions (Monahan et al., 1990; Pie et al., 1991; Monahan et al., 1992a, b). Monahan et al. (1992a, b) demonstrated that the rate of cholesterol oxidation in pork was greatly accelerated during storage following cooking and appeared to follow the same trend as lipid oxidation in general.

Published data were examined for specific trends of COPs accumulation produced by extreme heating (>150°C), but no trend was apparent. Only two groups of investigators reported the full complement for all 8 COPs in fresh, heated butter and fried Indian ghee (Pie et al., 1990; Kumar and Singhal, 1992).

Effects of storage and packaging

Park and Addis (1987) reported that the content of COPs in raw ground beef and turkey was essentially zero before storage. Monahan et al. (1992a) found that lipid oxidation in raw samples was low compared to processed samples (Park and Addis, 1987; Monahan et al., 1990) and may account for the low rates of cholesterol oxidation.

One freeze-dried pork sample was analyzed by Park and Addis (1987). Because this sample had been kept under abusive conditions, i.e., in the presence of air, uncontrolled relative humidity, laboratory fluorescent light and a very long storage time, the data obtained do not reflect commercial freeze-dried meat products. Nevertheless, the severity of cholesterol oxidation and the formation of triol, known to be one of the most atherogenic COPs, strongly suggests the need for investigations on COPs in commercial dehydrated and stored meat products.

Dry eggnog mix developed increasing levels of 7α- and 7β-hydroxycholesterol up to 80 days storage, after exposure to fluorescent light (Herian and Lee, 1985). Oxidized cholesterol in ppm have also been detected in long-term stored and cooked egg and dehydrated egg products (Tsai et al., 1979; Herian and Lee, 1985; Nourooz-Zadeh and Appelqvist, 1987). Because the major unsaturated fatty acids in egg are oleic acid and linoleic acid, it is likely that peroxy radicals of these unsaturated fatty acids attack cholesterol.

C-7 COPs as well as both epoxides were detected in raw veal, beef, pork and chicken and further confirmed by

combined GC-MS (Zubillaga and Maerker, 1991). Although chromatograms were not shown, the predominant species was reported to be 7-ketocholesterol, accounting for almost half of the total COPs, followed by 7β-OH, 7α-OH and both epoxides in order of decreasing concentration as indicated by relative peak height (Zubillaga and Maerker, 1991). The extent of cholesterol oxidation in both ground beef and turkey samples during storage was represented by total COPs level and related to percent of residual cholesterol content in each sample as well as corresponding TBARS. As expected, total COPs increased together with development of rancidity in both samples. However, at 0 days storage, the contents of COPs were essentially zero.

Luby et al. (1986) noted that only aluminum foil prevented cholesterol oxidation in butter exposed to fluorescent light after 15 days exposure. Margarine wrap opaque parchment, wet strength dry wax paper, and polyethylene film were not effective (Luby et al., 1986). They showed that photooxidation in butter would occur after prolonged exposure to light. Cholesterol oxidation occurred via singlet oxygen attack as well as free radical mechanisms. They had also demonstrated that it was a light-induced surface phenomenon, with COPs more concentrated at the surface than throughout the butter block (Luby et al., 1986).

Storage of unirradiated beef at 0-4 C for 2 wk increased the COPs content considerably (Hwang and Maerker, 1993). Unirradiated pork and veal during storage also showed an increase in most COPs, although increases were smaller than with beef. Irradiation of the meats followed by 2-wk storage at 1-4 C raised the COPs level considerably over that of unirradiated, stored samples, with exception of 4,6-cholestadien-3-one (Hwang and Maerker, 1993).

Effect of irradiation

Exposure to normal light sources or UV light or γ-radiation can initiate COPs formation in foods. After Luby et al. (1986) demonstrated that butter exposed to light accumulated cholesterol oxides, van de Bovenkamp et al. (1988) showed that irradiating dry egg yolk powder with UV for 3 wk greatly increase development of several COPs in foods. However, the egg yolk powder had been stored for 1 to 4 yr before exposure. Therefore, results also showed that storage had less effect on cholesterol oxides formation than did irradiation (van de Bovenkamp et al., 1988).

Researchers have sought a diagnostic test capable of detecting whether meats have been previously irradiated (Maerker et al., 1988; Zubillaga and Maerker, 1991). Studies revealed that exposure of cholesterol, in an aqueous environment, to gamma radiation led to generation of certain cholesterol oxidation products in ratios different from those produced by autoxidation (Lakritz and Maerker, 1989; Maerker and Jones, 1991). Specific cholesterol derivatives were 7-ketocholesterol, the α-epoxide and the β-epoxide. In irradiated aqueous sodium stearate dispersions of cholesterol, the ratio of 7-ketocholesterol to α-epoxide plus β-epoxide was $\approx$ 1 or below, while the ratio in autoxidation of similar dispersions was usually $\geq$ 6 (Lakritz and Maerker, 1989). In a study with cholesterol in aqueous suspensions of liposomes, the ratio of those compounds was $<$ 1.0 for irradiated samples, much lower than the ratio of 10, commonly produced by autoxidation (Maerker and Jones, 1991). Investigators suggested that the unusual relative amounts of these products may be characteristic of the effects of γ radiation. A-ring cholesterol derivatives such as 4-cholesten-3-one, 4,6-cholestadien-3-one, and 4-cholesten-3,6-dione were found to be generated from cholesterol in liposomes by exposure to γ-radiation (Maerker and Jones, 1993). These 3-keto derivatives of cholesterol are less polar (by TLC) than the parent sterol and hence more difficult to separate from neutral lipids than is cholesterol and/or its derivatives with hydroxy groups (Maerker and Jones, 1993).

Amounts of cholesterol oxidation generated by low-dose γ radiation were expected to be extremely small. It was important to develop methodology capable of distinguishing effects of autoxidation from those of radiation. The investigators' hypothesis that the ratio of COPs from γ radiation was different from those of autoxidation required testing in actual animal tissues. Therefore, the method to determine amounts of three compounds, 7-ketocholesterol, α-epoxide and β-epoxide, that would be normally found in meat and poultry was developed (Zubillaga and Maerker, 1991). The study was undertaken to determine if the findings from the model system (Moriarity and Maerker, 1990; Maerker and Jones, 1991) were applicable to actual meat systems. They were particularly interested in the A-ring oxidation products at a level as low as 10 ppb. The meat was irradiated at 4°C to a dose of 10 kGy. Although the COPs distribution patterns produced by γ-radiation in model systems have been reported to be different from those generated by autoxidation (Maerker and Jones, 1991, 1993), the same results were not observed between irradiated and unirradiated, fresh or stored meats (Zubillaga and Maerker, 1991; Hwang and Maerker, 1993). This dissimilarity of behavior of cholesterol in model membranes and in meat was probably attributable to the complexity of the meat systems (Hwang and Maerker, 1993). They suggested that energy from the low-dose γ-radiation might be distributed among other compounds besides cholesterol, such as proteins and other lipids. In addition, preformed oxides and natural antioxidant may have contributed to the complexity of the results.

The presence of A-ring oxidation products of cholesterol in foods has only been reported by Maerker's group (Moriarity and Maerker, 1990; Hwang and Maerker, 1993; Maerker and Jones, 1993). Other researchers also reported 4β-hydroxycholesterol in foods (Csiky, 1982; Sarantinos et al., 1993). These oxides, however, are naturally present in biological tissues (Peng and Taylor, 1984), low-density lipoproteins (Liu et al., 1991) and various mammalian organ tissues (Buchmann et al., 1987). Therefore, it may be impractical to use these A-ring oxidation products as markers for cholesterol oxidation or as indicators of "previous irradiation."

The most conspicuous finding was the very high level of COPs in dry egg yolk powder after 1 yr storage followed by irradiation with UV light for 3 wk (van de Bovenkamp et al., 1988). While food products would not be irradiated for 3 wk to reduce microbial growth or extend shelflife, it may be advisable to examine food for accumulation of COPs after commercial irradiation.

Prevention of cholesterol oxidation

The rate and extent of lipid oxidation are dependent on several factors, the most important being the level of polyunsaturated fatty acids present (Allen and Foegeding, 1981). Earlier studies concluded that triacylglycerols and phospholipids were important in the development of lipid oxidation in chicken (Igene et al., 1980) and fish (Tichivangana and Morrissey, 1985). The influ-

ence of triacylglycerols on development of rancidity was shown to depend on the degree of unsaturation (Igene et al., 1980). The polyunsaturated fatty acid content of muscle varies between species and decreases in the order fish > poultry > pork > beef > lamb (Pearson et al., 1977). Susceptibility to lipid oxidation was shown to be in the same order (Tichivangana and Morrissey, 1985). However it is generally accepted that phospholipids present in subcellular membranes (microsomes, mitochondria), rather than the triacylglycerols, are responsible for initial development of oxidized flavors in raw and cooked meat products during storage (Igene and Pearson, 1979; Younathan, 1985). In addition, subcellular membranes contain enzymatic systems capable of initiating peroxidative reactions (Slabyj and Hultin, 1984). Rupture of the compartmentalized cellular system by mincing or restructuring facilitates the formation of free radicals for propagation of oxidative reactions (Pearson et al., 1977). This provides further evidence that the intracellular phospholipid fraction is the primary lipid component involved in peroxidative changes.

Lipid oxidation is catalyzed by myoglobin, haemoglobin, cytochromes, nonheme iron and other heavy transition metals (Tappel, 1962; Igene and Pearson, 1979; Tichivangana and Morrissey, 1985). Apte and Morrissey (1987) clearly showed that the ferritin fraction contributed significantly to lipid oxidation in heated meat systems. The susceptibility of muscle to lipid oxidation is also influenced by the presence of antioxidants. Dietary vitamin E (α-tocopherol) supplementation improved the oxidative stability of muscle from fish (Frigg et al., 1990), chicken (Brekke et al., 1975; Marusich et al., 1975; Lin et al., 1989), pork (Buckley and Connolly, 1980; Monahan et al., 1990) and veal (Engeseth et al., 1993). The application of antioxidants to extend shelf-life of meat products has been reviewed by Gray and Pearson (1984). Considerable interest has also been expressed in the use of antioxidants incorporated into animal diets and their effects on meat quality (Pearson et al., 1977; Gray and Pearson, 1984).

Monahan et al. (1990) investigated the effects of dietary α-tocopherol supplementation for 2 wk prior to slaughter, on plasma and muscle α-tocopherol levels and on oxidative stability of raw and cooked pig muscle during refrigerated storage at 4°C. They found that the α-tocopherol level in plasma and muscle of pigs on supplementary diet were about 2.5-fold higher than those of controls. They claimed that this increase in level of α-tocopherol improved the oxidative stability for storage at 4°C up to 8 days.

Later they (Monahan et al., 1992a) compared the effects of dietary fat (soya oil and tallow) on stability of meat. Pigs fed soya oil were more susceptible to lipid oxidation than those fed a tallow diet (Monahan et al., 1992a). However, α-tocopherol supplementation (200 mg/kg feed) significantly increased the oxidative stability of both groups (Monahan et al., 1992a, b).

Engeseth et al. (1993) investigated the effects of supplementary α-tocopherol (500 mg/kg feed) on stability of veal and concluded that cholesterol oxidation could be controlled by α-tocopherol. The 65% reduction in oxides attributed to α-tocopherol supplementation seemed misleading for several reasons. Samples had been frozen several months prior to analysis. COPs could have been produced from artifacts in the analytical procedure because isolation involved two column separations and at least two drying/freezing periods. Moreover, data for day 0 storage showed greater concentrations than that on day 4.

Morgan and Armstrong (1987) showed that elevated temperature and pro-oxidizing agent (H_2O_2) markedly increased cholesterol-5,6-epoxides production. However, the antioxidants butylated hydroxyanisole and butylated hydroxytoluene displayed an inhibitory effect (Morgan and Armstrong, 1987).

Several researchers have suggested that prevention of cholesterol oxidation in processed foods should be similar to procedures to prevent lipid oxidation. Those suggestions include:

1. Feeding/ incorporating antioxidants into live animal or raw meat before processing (Buckley and Connolly, 1980; Park and Addis, 1986b; Monahan et al., 1990).

2. Low temperature or minimum temperature processing (Tuohy and Kelly, 1989; Chan et al., 1993).

3. Packaging with exclusion of O_2 (Chan et al., 1993).

4. Storage conditions including low temperature, low light (Luby et al., 1986).

SUMMARY

ALTHOUGH MANY FOODS are somewhat resistant to autoxidation of cholesterol, potential for cholesterol oxidation exists. Research on occurrence of COPs in foods has been based on reliable methods only since the mid 1980s. Their study is still limited because there is no existing standard method which includes adequate accuracy, precision, selectivity, consistency and speed. A thorough analysis of all cholesterol-containing foods should be conducted. A high priority should be placed on such investigations along with suitable toxicological studies. Although some published values for cholesterol and COPs appear reasonable, there is an urgent need for repeated analyses of many foods by standard methods of analysis. Until this is done, many values for cholesterol and COPs must be considered as approximations. Because total elimination of such compounds from the human diet may be impossible, investigations concerning toxicological effects of long-term exposure to low dietary concentrations of atherogenic sterols are also needed.

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Ms received 9/10/94; revised 4/22/95; accepted 5/18/95.

Display Life and Internal Cooked Color of Ground Beef from Vitamin E-Supplemented Steers

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ABSTRACT

The retail display life of fresh ground meat and the internal color of patties cooked to four endpoint temperatures (55, 65, 71, and 77°C) were determined for ground beef (10% fat) from vitamin E-supplemented (500 and 2000 IU) or nonsupplemented steers. Vitamin E supplementation increased ($P < 0.05$) the retail display life of the meat by 12 and 42 hr for 500 and 2000 IU, respectively. Vitamin E did not affect ($P > 0.05$) internal cooked color or expressible juice color of the patties, and it did not cause premature browning or persistent redness. Increased levels of vitamin E supplementation decreased ($P < 0.05$) TBA values but had no effect on total reducing activity or oxidation-reduction potential.

Key Words: beef, internal color, display-life, vitamin E, dietary supplement

INTRODUCTION

DISPLAYING MEAT CUTS under high-intensity lights, as in retail cases, accelerates formation of undesirable brown, metmyoglobin, color (Faustman and Cassens, 1990). Retailers lose more than a billion dollars every year due to discoloration of meat cuts prior to microbiological spoilage (Hermel, 1993). Research has focused on the feasibility of supplementing the diet of cattle with vitamin E in order to lessen such losses. Vitamin E supplementation has been shown to result in an extension of 1.6–5 days of retail display life without compromises in microbiological quality (Arnold et al., 1992, 1993b; Cabedo, 1994).

Since vitamin E feed supplementation affects retail display color stability, it is important to determine whether vitamin E may also affect cooked color. The internal color of ground beef patties usually is related to endpoint temperatures. Patties cooked to 65°C (medium-rare) have a red-pink interior; those cooked to 71°C (medium), a tan interior; and those cooked to 77°C (well-done), a brown interior (Marksberry et al., 1993). However, deviations from such "typical" cooked colors have been reported.

In one type of atypical cooked color, premature browning, patties have a brown interior at endpoint temperatures (<55°C) where they should still be red or pink (Marksberry, 1990; Hague et al., 1994; Warren et al., 1995a). This condition represents a potential safety hazard, because patties appear to be cooked thoroughly, yet any pathogens that may have been present may be still viable. Premature browning is thought to be related to the oxidation state of the internal myoglobin prior to cooking (Warren et al., 1995b). Patties with their interior myoglobin in an oxidized state immediately prior to cooking are more likely to be premature brown, while patties with interior myoglobin in a reduced state prior to cooking are more likely to be "normal." Machlik (1965) showed that the oxidation state of myoglobin influenced its rate of denaturation during heating. The order of stability to denaturation was deoxymyoglobin > oxymyoglobin > metmyoglobin. These denaturation rates agreed with observations recorded by Warren et al. (1995b).

In another type of atypical cooked color, persistent pinkness, patties retain an internal pink color at medium (Van Laack et al., 1994) and well-done temperatures (Mendenhall, 1989). This condition has been attributed to the increased resistance of myoglobin to denaturation in high-pH meat (Trout, 1989; Van Laack et al., 1994), as well as endogenous factors such as ammonia, nitrite, and carbon monoxide (Shaw et al., 1992; Cornforth et al., 1986).

Our objectives were to determine the effects of vitamin E feed supplementation on ground beef retail color stability and cooked color development.

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MATERIALS & METHODS

Meat

Knuckles (quadriceps muscles, NAMP #167 (NAMP, 1988)) from 36 Holstein steers were obtained from the University of Wisconsin-Madison. The meat represented two replications, separated by 45 days, of 18 steers each; six received no supplemental Vitamin E, six received 500 IU, and six received 2000 IU daily for ≈120 days prior to slaughter. The meat was processed, vacuum-packaged, and shipped fresh to Kansas State University by Packerland (Green Bay, WI). The cuts were stored at 2°C until 17 days postslaughter.

The knuckles were trimmed of excess fat and superficial muscles (nonquadricep) and coarsely ground (1.27 cm plate). Fat trimmed from the knuckles and inside rounds of the same animal was frozen at −20°C, finely ground (0.32-cm plate), and added to the lean to achieve ≈10% fat. The mixture was blended in a Hobart mixer (Model 4732, Troy, MI) for 2 min and then twice ground through a 0.32-cm plate. Visual-display and initial microbiological samples were removed, and the remainder of the meat was formed into 113-g patties using a Hollymatic pattymaker (Jet Flow Super, Model 54, Countryside, IL). The patties were frozen at −40°C, vacuum-packaged, and stored at −20°C until used.

Display color stability

Trays were used for display color stability of the fresh ground beef to simulate what consumers encounter at retail stores. Immediately after grinding, 340.5g of meat was placed onto a 10.2 × 10.2 cm polystyrene tray (Mobil Chemical Packaging Department, Temple, TX), distributed evenly, and wrapped with polyvinyl chloride film (Borden Packaging and Industrial Products, North Andover, MA).

Samples were arranged randomly in a 0–2°C commercial, open-topped display cabinet (Model DMF8, Tyler Refrigeration Corporation, Niles, MI), programmed for one daily defrost cycle, and displayed continuously under 1614 lux of Phillips 40W DLX warm white fluorescent lights. Samples were rotated twice daily to minimize any light and temperature fluctuation effects within the cabinet.

Samples were scored to the nearest 0.5 by six panelists, trained to recognize the extremes and midpoint, using a 5-point scale (1 = bright red, 2 = red, 3 = reddish-brown, 4 = moderately brown, 5 = very brown). Panelists evaluated samples before display (0 hr) and at 16, 23, 44, 68, and 92 hr after initial display.

At the same times as visual scoring, samples were scanned with a LabScan 6000 Spectrocolorimeter (2-cm-diamaperture. Hunterlab, Reston, VA). Average CIE L*, a*, b* (Illuminant A), a*/b* ratio, saturation index, and hue angle values were calculated for each display package. Reflectance at 630/580 nm, a measure of decreasing redness as ratio decreases, also was determined.

Microbiology

Microbiological samples were collected before display (0 hr) and at 44 and 92 hr after initial display and analyzed for total aerobic plate counts (APCs) to determine whether microorganisms affected display

Table 1—Chemical analysis[d] of ground beef containing three levels of vitamin E

Trait	Vitamin E supplementation, IU 0	500	2000	SE
Fat, %	8.7	8.6	8.5	0.1
Oxidation-reduction potential, mV	−72.8	−67.7	−71.9	1.4
Total reducing activity	0.30	0.29	0.28	0.01
Raw patty pH	5.63[ab]	5.65[a]	5.62[b]	0.01
TBA value, µg TBA reactive/g sample	1.3[a]	0.8[b]	0.5[c]	0.1
α-Tocopherol, µg/g fresh meat	1.22[a]	2.43[b]	5.31[c]	0.18

[a-c] Means within a row with a different letter are different ($P < 0.05$).
[d] Data were pooled for replication.

color stability. The 0 hr samples were collected immediately after grinding. The 44- and 92-hr samples were taken from extra packages that were treated as described. From each sample, 25 g of surface meat was weighed into a stomacher bag (Spiral Biotech Inc., Bethesda, MD) and mixed in a Stomacher-400 (Tekmar Company, Cincinnati, OH) for 2 min with 30 mL of sterile 0.1% peptone diluent. APCs were determined by plating 1 mL of the sample homogenate and appropriate 10-fold dilutions using the pour plate method (Vanderzant and Splittstoesser, 1992). Plates were incubated for 24 hr at 32°C before being enumerated to determine APC/g for sample.

Internal cooked color

All patties were frozen for ≈230 days prior to cooking (described below). One set of frozen, vacuum-packaged patties was thawed overnight at 3°C and then cooked. These patties developed a premature brown color. Another set of patties was thawed overnight at 3°C and then held at 22°C for 4.5 hr to further reduce the pigment to deoxymyoglobin. Prior to cooking, patties were stored at 3°C for 2 hr. These patties had a normal cooked color.

All patties were cooked immediately after removing from vacuum packages on a preheated (162.8°C) electric griddle (Presto, Model 0703202) to either 55, 65, 71, or 77°C. Patties were turned every 30 sec and were removed from the griddle 1.5°C before reaching the endpoint temperature. Near the end of cooking, internal temperature was measured by intermittently inserting a 20-gauge hypodermic probe-type thermocouple connected to a Doric temperature recorder (Trendicator 410A, San Diego, CA) into the geometric center of the patty at a 45° angle. Cooking times did not differ ($P>0.05$) between treatments (results not shown).

Some patties were cooled for 1–3 min at room temperature after cooking; others were cooled 7–10 min postcooking. The two different times of cooling were used because expressive juice was not measured for all patties cooked. The patties were sliced vertically, and the internal color was ranked visually to the nearest 0.5 by one panelist on a 5-point color scale (Marksberry et al., 1993). Half of the patty was sliced horizontally and placed on plastic wrap; the color was measured instrumentally as described.

Expressible juice color

The patties cooled 1–3 min following cooking were used to determine expressible juice color. The juice was pressed from one-half of the patty, with a hand-held patty former, and the other half was used for instrumental color measurement. A small volume of juice supernatant was transferred to a clear plastic tissue-culture cell, and L*, a* and b* values were measured using a Minolta Chroma Meter (Model CR-200). Expressible juice color also was visually scored to the nearest 0.5 by one panelist on a 5-point scale (Marksberry et al., 1993).

Chemical analyses

All analyses were performed on patties that had been stored at −20°C for ≈245 days. To measure pH, 10 g raw meat and 100 mL distilled water were blended for 30 sec using a Lab Blender 400 Stomacher (Tekmar). The pH was measured using a Fisher Accumet pH meter (Model 620, Orion Ross Combination Electrode, Fisher Scientific, Pittsburgh, PA). Fat content of the raw patties was determined with a Foss-Let Fat Analyzer (Foss Electric, Hillerod, Denmark) using AOAC (1990) methods. The method of Cornforth et al. (1986) was used to measure oxidation-reduction potential. Briefly, 10 g of unthawed whole muscle, trimmed from the knuckles prior to coarse grinding, was blended with 15 mL of 0.1 M sodium carbonate using a Brinkman Polytron (setting 5.5). Oxidation-reduction potential was measured under nitrogen using a Fisher Accumet pH meter (Model 925, Fisher Scientific) with a platinum redox and a silver/silver chloride reference electrode (Orion, Inc., Cambridge, MA). Total reducing activity was determined using the method of Lee et al. (1981). 2-Thiobarbituric acid (TBA) values were determined using the modification by Kuntapanit (1978) of the method of Witte et al. (1970). For α-tocopherol analysis, vastus intermedius muscle samples taken prior to grinding were frozen in liquid nitrogen, then powdered by crushing between complementary stainless steel plates. The concentration of α-tocopherol in 1 g of the powder then was determined using the method of Arnold et al. (1993b).

Statistics

Significance was established at $p \leq 0.05$. The instrumental data for display and normal cooked color data were analyzed as split-plots using the General Linear Model procedure of the Statistical Analysis System (SAS Institute, Inc., 1988). The data were blocked by replication with the whole-plot factor being vitamin E level and the split-plot factor either hour of display or endpoint temperature. The prematurely brown patties were analyzed using a split-split-plot. The whole-plot factor was vitamin E level, the split-plot factor endpoint temperature, and the split-split-plot factor was length of time at room temperature postcooking. The visual panelist data were analyzed as a stacked plot. Replication was a block and the error terms were replication × hour, replication × hour × panelist, replication × vitamin E × hour, and replication × hour × panelist × vitamin E. The chemical analysis data were analyzed as a randomized block design with replication as the block and vitamin E as the treatment. Differences among means were determined using least square means.

RESULTS & DISCUSSION

PATTIES for each of the vitamin E treatments were formulated to the same percentage fat, and vitamin E level did not affect total reducing activity or oxidation-reduction potential (Table 1). Vitamin E also had no effect on the $\log_{10}$ total APCs of the display samples at either 0, 44, or 92 hr ($\log_{10}$ 3.0, 5.1 and 5.1, respectively). Arnold et al. (1992, 1993b) and Cabedo (1994) also have shown that vitamin E supplementation did not affect the growth of microorganisms. Vitamin E level had an effect ($P < 0.05$) on raw patty pH values; however, this effect showed no clear trend, and the differences were not substantial. Chromatographic analyses indicated that the quantity of α-tocopherol in the samples increased with vitamin E level (Table 1).

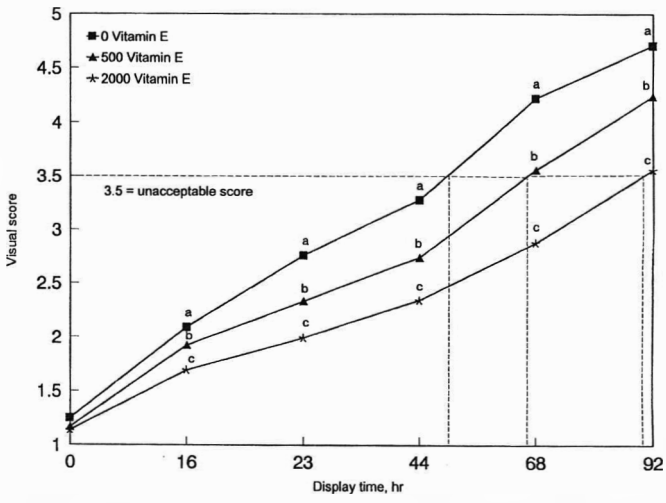

Fig. 1—Effect of vitamin E on visual color score of ground beef during lighted display at 0°C. Visual score: 1 = bright red, 2 = red, 3 = reddish-brown, 4 = moderately brown, 5 = very brown. Data were pooled for replication. SE = 0.05. Data points within each hour with a different letter are significant ($P < 0.05$).

Display color stability

Visual panel scores were affected by vitamin E. By 16 hr the panelists consistently ranked the samples with 2000 E as most red and 0 E as least red (Fig. 1). Based on a score of 3.5 considered unsalable, the 0 E samples were salable ≈48 hr, the 500 E samples 60 hr, and the 2000 E samples about 90 hr. Instrumental values for a*, a*/b* ratio, saturation index, and hue angle exhibited a vitamin E × hour interaction (Table 2). The level of vitamin E had no effect ($P > 0.05$) on values of color parameters at 0 and 16 hr. By 23 hr, the 500 and 2000 E samples had higher a* and saturation index values ($P < 0.05$) than the 0 E samples, and by 44 hr, the vitamin E samples also had higher a*/b* ratios and lower hue angles ($P < 0.05$) than controls. The 500 and 2000 E samples differed ($P < 0.05$) only at 68 and 92 hr, with the 2000 E samples having a higher a*/b* ratio and lower hue angle ($P < 0.05$) than 500 E samples. L*, b*, and 630/580 nm values were independent of vitamin E level ($P > 0.05$). The absence of an effect of vitamin E on reflectance values at 630/580 nm indicated that this parameter did not accurately describe differences between treatments. However, these values were dependent ($P < 0.05$) on the hour of display. L* and b* values decreased ($P < 0.05$) up to 68 hr, and reflectance measurements at 630/580 nm decreased ($P < 0.05$) continuously during display (Table 3).

Samples with vitamin E retained a more intense red color longer than those without vitamin E. Differences between the 500 and 2000 E samples were instrumentally apparent only in a*/b* and hue angle values on and after 68 hr display. Vitamin E did not prevent discoloration of samples. Instead, it affected the rate at which samples discolored (Fig. 1). Lanari et al. (1994) deduced from kinetic analyses that vitamin E acted to increase the rate at which deoxymyoglobin was converted to oxymyoglobin, while also decreasing the rate of oxymyoglobin autoxidation.

The observed increase (≈0.5 days for 500 E and 1.75 days for 2000 E) in retail display color stability was expected. Cabedo (1994) reported a 3.5-day extension for ground beef from animals supplemented with 1000 and 2000 IU. Differences in retail display life extensions may be attributed to the amount of vitamin E in the muscle, muscle fiber type, retail case temperature, defrost cycles, or light intensity. The retail display extension in our study was not as long as the 5-day maximum Arnold et al. (1992, 1993b) reported for intact cuts of meat. This would be expected, because grinding exposes more cellular surface area to oxidative damage.

Vitamin E affected the TBA values of the patties (Table 1); values were reduced as vitamin E increased. The decrease in TBA values with increasing vitamin E concentration could explain why the display samples exhibited different color stabilities. Other researchers have found that supplementary vitamin E effectively reduced lipid oxidation (Arnold et al., 1992, 1993b; Buckley et al., 1989; Faustman et al., 1989; Lanari et al., 1994). Arnold et al. (1993a) and Faustman et al. (1989) reported that the amount of lipid oxidation positively correlated with the percent metmyoglobin in a sample. Thus, the amount of lipid oxidation observed for the 0, 500, and 2000 E samples in our study may have been related to differences in display color stability.

Table 2—Changes[d] in color parameters during display for ground beef containing three levels of vitamin E

Traits/vitamin E level	Display time, hr 0	16	23	44	68	92	SE
a*							
0 IU	37.6	29.5	26.4[a]	20.5[a]	14.4[a]	10.9[a]	0.8
500 IU	39.8	32.5	30.9[b]	25.6[b]	19.7[b]	15.1[b]	0.6
2000 IU	39.0	31.7	30.5[b]	26.1[b]	20.7[b]	17.4[b]	0.7
a*/b*							
0 IU	1.29	1.24	1.22	1.05[a]	0.81[a]	0.64[a]	0.03
500 IU	1.33	1.29	1.28	1.19[b]	1.00[b]	0.81[b]	0.02
2000 IU	1.32	1.30	1.29	1.23[b]	1.09[c]	0.99[c]	0.02
Saturation index							
0 IU	47.5	37.6	34.2[a]	28.3[a]	23.2[a]	20.6	0.9
500 IU	50.0	41.2	39.3[b]	33.5[b]	27.9[b]	23.8	0.6
2000 IU	49.8	41.3	39.3[b]	34.4[b]	29.0[b]	25.3	0.7
Hue angle							
0 IU	37.8	38.9	39.5	43.6[a]	51.2[a]	57.4[a]	0.8
500 IU	37.0	37.7	38.1	40.2[b]	45.2[b]	51.1[b]	0.5
2000 IU	37.2	37.5	37.8	39.1[b]	42.7[c]	45.4[c]	0.6

[a-c] Means in a column and for the same trait with different letters are different ($P < 0.05$).
[d] Data were pooled for replication.

Table 3—Changes[g] in color parameters of ground beef over retail display time

Trait	Display time, hr 0	16	23	44	68	92	SE
L*	52.1[a]	48.9[b]	47.8[c]	46.6[d]	45.7[e]	46.2[e]	0.4
b*	30.1[a]	25.9[b]	23.8[c]	20.8[d]	19.0[e]	17.8[e]	0.8
630/580 nm	6.6[a]	4.1[b]	3.6[c]	2.5[d]	1.6[e]	1.2[f]	0.1

[a-f] Means within a row with different letters are different ($P < 0.05$).
[g] Data were pooled for replication and vitamin E.

Table 4—Cooked color measurements[f] of patties that developed a normal cooked color at four endpoint temperatures

Trait	Temperature, °C 55	65	71	77	SE
L*	55.3[a]	57.3[b]	57.1[b]	56.9[b]	0.3
a*	22.4[a]	19.3[b]	16.3[c]	13.2[d]	0.4
b*	17.8[a]	17.0[b]	16.2[c]	15.7[c]	0.2
a*/b*	1.26[a]	1.14[b]	1.01[c]	0.85[d]	0.02
Saturation index	28.6[a]	25.7[b]	23.0[c]	20.5[d]	0.4
Hue angle	38.6[a]	41.6[b]	45.0[c]	49.9[d]	0.5
630/580 nm	2.4[a]	2.0[b]	1.7[c]	1.4[d]	0.1
Visual score[e]	1.6[a]	2.1[b]	3.0[c]	4.3[d]	0.1

[a-d] Means within a row with different letters are different ($P < 0.05$).
[e] Visual score ranges from 1-pink to 5-brown (Marksberry et al., 1993).
[f] Data were pooled for replication and vitamin E.

Cooked color

Patties with normal color. Vitamin E had no effect ($P > 0.05$) on patty internal cooked color when a normal (i.e., decreasing redness with increasing endpoint temperature) cooked color pattern occurred. As expected, the internal color was affected ($P < 0.05$) by endpoint temperature (Table 4). With the exception of L* and b* values, all color parameters, including visual scores, were different ($P < 0.05$) at each endpoint temperature. L* values increased up to 65°C, and b* values remained constant between 71 and 77°C. These results were consistent with those reported by Warren et al. (1995a).

Prematurely brown patties. Vitamin E had no effect ($P > 0.05$) on premature browning, i.e., all patties thawed without an anaerobic reduction step turned brown at a lower than expected temperature. There was a temperature by vitamin E interaction ($P < 0.05$). However, L*, a*, b*, 630/580 nm, and saturation index values at two of the four temperatures were affected when cooked color was measured at 7–10 min postcooking rather than at 1–3 min postcooking (Table 5). At 55°C, patties had a darker red internal appearance when color was measured 1–3 min postcooking as compared to 7–10 min. At 77°C, the patties at 7–10 min were darker red. Visual scores recorded 1–3 min after cooking were lower (more redness) than those recorded 7–10 min postcooking for both 55 and 65°C (Table 5). When internal color was measured 7–10 min postcooking, visual scores increased as endpoint temperature increased (Table 5). The same was true for patties measured 1–3 min postcooking, except there were no differences in visual scores of 71 and 77°C patties. Differences in color traits of the 1–3 min and 7–10 min patties could have been due to additional denaturation of the pigment. The instrumental values were comparable to those previously reported for prematurely brown patties by Hague et al. (1994) and Warren et al. (1995a).

Comparisons between premature brown and normal patties. Normal patties had higher L*, a*, b*, 630/580 nm, a*/b*

Table 5—Color traits of patties that developed a premature brown color when cooked to four endpoint temperatures and evaluated at 1–3 and 7–10 min postcooking[e]

Trait	55°C		65°C		71°C		77°C		SE
	1–3	7–10	1–3	7–10	1–3	7–10	1–3	7–10	
L*	53.5[a]	54.7[b]	54.8[a]	55.6[b]	55.6	55.3	55.4	54.9	0.3
a*	15.9[a]	14.7[b]	13.1	12.9	11.7	12.1	11.0[a]	11.9[b]	0.2
b*	17.0[a]	16.0[b]	14.9	14.9	14.3	14.6	14.1[a]	15.1[b]	0.1
a*/b*	0.93	0.91	0.88	0.87	0.82	0.83	0.78	0.79	0.01
Saturation index	23.2[a]	21.8[b]	19.9	19.7	18.5	18.9	17.9[a]	19.2[b]	0.2
Hue angle	47.0	47.8	48.8	49.2	50.7	50.5	52.0	51.8	0.3
630/580 nm	1.6[a]	1.5[b]	1.4	1.4	1.3	1.3	1.2	1.3	0.1
Visual score[c]	2.8[w]	3.9[w]	3.4[w]	4.1[x]	4.3[y]	4.4[y]	4.9[z]	4.5[y]	0.1
Juice visual score[d]	2.1[w]	—	3.0[x]	—	3.8[y]	—	4.2[z]	—	0.1
Juice L*	70.1[w]	—	73.5[x]	—	76.2[y]	—	76.2[y]	—	0.7
Juice a*	12.1[w]	—	10.9[w]	—	7.1[x]	—	5.6[y]	—	0.5
Juice b*	14.9[w]	—	17.2[x]	—	18.7[y]	—	19.3[y]	—	0.5

[a-b] Means within a trait at a particular time with different letters are different ($P < 0.05$).
[c] Visual score ranges from 1-pink to 5-brown (Marksberry et al., 1993).
[d] Expressible juice visual score ranges from 1-red to 5-yellow (Marksberry et al., 1993).
[e] Data were pooled for replication and vitamin E.
[w-z] Means within a trait and time postcooking with different letters are different ($P < 0.05$).

ratio, saturation index, and ΔE values and lower hue angle values (Table 6). As patties were cooked to a well-done state (i.e., 77°C), the internal cooked color of normal and prematurely brown patties became similar. Patties with normal cooked color visually had a much redder interior at the lower endpoint temperatures. Even at 71°C, the USDA (1992) and FDA (1993) recommended endpoint temperature for ground beef patties (Hague et al., 1994), differences between normal and prematurely brown patties were apparent, especially with regard to a* and saturation index values.

Comparison between a common set of patties handled such that they developed different cooked color adds a new perspective to the cause of premature browning in ground beef. The degree of oxidation of the interior myoglobin of the prematurely brown and normal patties was not measured quantitatively but some general observations were noted. The patties that were prematurely brown had a brown purge and patty color, indicative of metmyoglobin, when vacuum-packaged. They also had a brown interior prior to cooking. The patties that developed normal cooked color had a purplish purge and patty color, indicative of deoxymyoglobin, when vacuum-packaged; they had a deep red-purple interior. Such observations may be related to the internal myoglobin oxidation state of the patties prior to cooking. All patties, both prematurely brown and normal, were frozen in an oxymyoglobin state. By vacuum-packaging the samples, the partial pressure of oxygen in the packages was lowered to a level where metmyoglobin formation was promoted (Ledward, 1970). By allowing the patties to reduce at room temperature, the enzymes naturally present in the meat may have utilized most of the remaining oxygen, thus lowering the oxygen partial pressure (Ledward, 1985; Echevarne, et al., 1990) and resulting in deoxymyoglobin formation (Faustman and Cassens, 1990). Because metmyoglobin is more sensitive to heat denaturation than deoxymyoglobin (Machlik, 1965), premature browning would have resulted in those patties not being reduced anaerobically. Warren et al. (1995b) also showed that patties with oxidized pigments were browner than those in the ferrous form, thus supporting this hypothesis of the premature browning mechanism.

Warren et al. (1995b) also reported that total reducing activity was related to premature browning. Our study shows that premature browning may be independent of total reducing activity. However, meat age and processing conditions may also be factors.

Expressible juice

Expressible juice was only measured for the first complete set of patties cooked, including both replications. These patties were handled such that they were prematurely brown. Statistical analysis indicated that vitamin E did not affect ($P > 0.05$) expressible juice color. Thus, this trait was not measured for the rest of the patties, including those that had normal color. The expressible juice data that were collected indicated that the lightness and yellowness of the juice (L* and b* values) increased up to 71°C (Table 5). Juice had the same redness (a* values) at 55 and 65°C but became less red and lighter thereafter (Table 5). The trends and magnitudes of values we observed differed from those reported by Hague et al. (1994). However, juice visual scores from both studies closely paralleled. Differences in expressible juice L*, a*, and b* values in the two studies may have been attributable to the depth of liquid measured, the length of time the patty remained at room temperature prior to juice expulsion, or treatment of the meat.

Table 6—Cooked color trait differences[c] (normal minus prematurely brown) at four endpoint temperatures

Trait	Temperature, °C			
	55	65	71	77
L*	0.6	1.7	1.8	2.0
a*	7.7	6.4	4.2	1.3
b*	1.8	2.1	1.7	0.6
a*/b*	0.35	0.27	0.18	0.06
Saturation index	6.8	6.0	4.1	1.3
Hue angle	−9.2	−7.6	−5.5	−1.9
630/580 nm	1.0	0.6	0.4	0.1
Visual score[a]	−2.3	−2.0	−1.4	−0.2
ΔE[b]	7.9	7.0	4.8	2.5

[a] Visual score ranges from 1-pink to 5-brown (Marksberry et al., 1993).
[b] ΔE (normal-prematurely brown)$=[(\Delta L^{*2}) + (\Delta a^{*2}) + (\Delta b^{*2})]^{1/2}$
[c] Data were pooled for replication.

CONCLUSION

VITAMIN E SUPPLEMENTATION was effective in increasing retail display life of ground beef. Based on visual panel scores, supplementation with 2000 IU vitamin E, as compared to 500 IU, was more effective in increasing display color stability and reducing retail losses due to discoloration. Vitamin E supplementation did not affect internal cooked color or expressible juice color of patties. It did not cause premature browning or persistent redness. Premature browning would occur in patties containing vitamin E, if metmyoglobin were present in the patty interior at the time of cooking.

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—Continued on page 1196

Vacuum-Packaged Precooked Pork from Hogs Fed Supplemental Vitamin E: Chemical, Shelf-Life and Sensory Properties

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ABSTRACT

Precooked longissimus chops and semimembranosus/adductor roasts from pigs (n = 30) given no supplemental vitamin E (CON) or supplemented with 100 mg vitamin E/kg diet (VITE) were evaluated for lipid oxidation, microbial growth, sensory characteristics, cooking/storage losses and reheating losses. Chops and roasts were vacuum packaged, precooked to 60°C and stored at 2°C for 0, 7, 14, 28, or 56 days. Lipid oxidation was lower in VITE chops and roasts than in CON chops and roasts. Off-flavor intensity scores were more acceptable and storage/cooking losses were lower for VITE roasts than for CON roasts. Supplementation of vitamin E in a swine diet provided added protection against lipid oxidation and precooking pork under vacuum provided a palatable product with a shelf-life of ≥ 56 days.

Key Words: precooked pork, vitamin E, shelf-life, vacuum packaged

INTRODUCTION

A PROBLEM associated with precooked/stored/reheated meat is warmed-over flavor (WOF), caused by oxidation of lipids (Tims and Watts, 1958). Such oxidation greatly reduces consumer acceptability because of associated rancid flavors (Cross et al., 1987). Warmed-over flavor is an important factor in manufacturing and marketing precooked meat products.

Dietary vitamin E (α-tocopherol) may be useful as an antioxidant for meat that is to be precooked. Vitamin E inactivates free radicals in cell membranes, thus inhibiting oxidation of phospholipids, the primary source of WOF (Coelho, 1991). Previous studies have shown that lipid oxidation was inhibited in cooked and stored poultry (Lin et al., 1989; Ajuyah et al., 1993) and pork (Monahan et al., 1990a,b, 1992b) when the meat was from animals fed supplemental vitamin E. Successful inhibition of WOF by dietary supplementation of vitamin E would enable production of precooked meat products with acceptable shelf-life and sensory characteristics.

Our objective was to determine the influence of supplemental vitamin E, fed to pigs for 84 days prior to slaughter, on lipid oxidation, shelf-life and sensory characteristics of pork precooked using cook-in-bag technology.

MATERIALS & METHODS

Feeding regimen

The dietary treatments and feeding period of the pigs were reported in Cannon et al. (1995). Briefly, 30 crossbred pigs were assigned to five pen blocks based on weight. Within each block, pigs were randomly allotted to one of two treatment groups: (1) a control diet containing no supplementary vitamin E (CON) and (2) a diet formulated to contain 100 mg/kg diet supplementary vitamin E (VITE). After an 84 day feeding period, pigs were slaughtered using commercial procedures.

Precooked chop study

At 4 days postmortem, loins from the right side of each carcass were removed, deboned and trimmed to no more than 0.31 cm of external fat. Each loin was sliced into 2.54 cm chops and randomly assigned to five vacuum storage times (three chops/storage time) of 0, 7, 14, 28, or 56 days. Three chops/storage group were packaged together, under vacuum (−0.8 bar), in cook-in-bags (CN-530; oxygen transmission rate, 20 cc/m²/24 hr, 1 atm @ 22.8°C and 0% RH; Cryovac Division, W.R. Grace and Company, Ft. Worth, TX). Chops were steam-cooked in a commercial oven (Alkar Model 450, Alkar, Lodi, WI) to internal temperature 60°C, showered (21°C) for 10 min and then stored in the absence of light at 2°C for the specified storage period. Loin chops assigned to the 0 days storage period were evaluated after a 24 hr cooling period. Lipid oxidation determination, total plate count (TPC), sensory analysis, pH, cooking/storage losses and reheating losses were evaluated at each storage time. Cooking/storage losses were determined from weights recorded prior to packaging and immediately after removal from the package.

Lipid oxidation was determined using thiobarbituric acid (TBA) analysis procedures of Salih et al. (1987), with the modification of 5% (w/v) aqueous trichloroacetic acid as the extraction solvent instead of perchloric acid (Raharjo et al., 1993). Results were expressed as TBA values (mg malonaldehyde/kg wet tissue).

Duplicate samples for microbial evaluation were obtained by aseptically removing 10 g tissue samples and placing them into stomacher bags containing 90 mL of a sterile 0.1% peptone water solution. Samples were placed into a stomacher apparatus (Stomacher Lab-Blender 400, Tekmar Company, Cincinnati, OH) and homogenized for 2 min. Appropriate serial dilutions were made in sterile peptone water and 0.1 mL of each diluent were spread onto total plate count (TPC) agar (Bacto Nutrient Agar, Difco Laboratories, Detroit, MI). Plates were incubated at 25°C for 48 hr and bacteria colonies were counted. The pH of the blended samples was determined at each sampling time using an Accumet pH meter 50 (Fisher Scientific, Pittsburgh, PA).

Chops for sensory evaluation were reheated on Farberware open-hearth grills (Farberware Model 155N, Walter Kidde, Inc., Bronx, NY) to internal temperature 70°C monitored by a thermocouple (Atkins Technical Inc., Gainesville, FL). Chops were turned once at 35°C to prevent charring. Immediately after reheating, cubed portions from each chop were served to a 6-member trained sensory panel for evaluation of tenderness, juiciness, pork flavor intensity and off-flavor intensity. The taste panel members had previous sensory evaluation experience. The panel was given an additional 4-day training period where they evaluated precooked pork which had been stored for extended periods of time, to

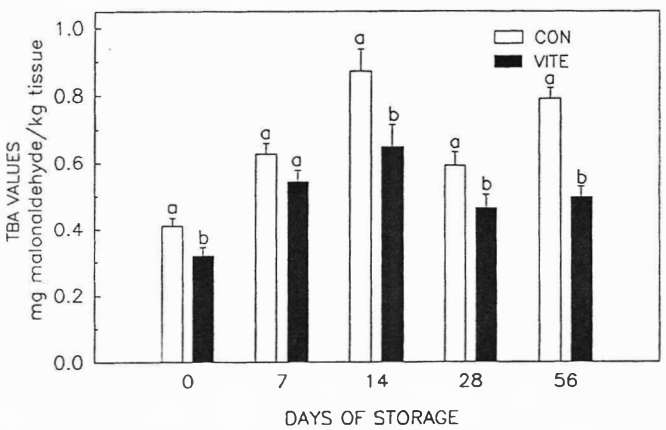

Fig. 1—TBA values for precooked chops from controls and pigs supplemented with vitamin E. The following model effects were observed: Treatment = *, Storage = * and Treatment × Storage interaction = *; where * = $P < 0.05$ and NS = not significant. [a,b]Treatment means within the same storage period lacking a common superscript letter differ ($P < 0.05$).

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Table 1—Sensory properties of precooked chops stored 7 to 56 days from controls and pigs supplemented with vitamin E[a]

Trait[b] and treatment[c]	Days 7	14	28	56	Model effects[d]
Juiciness					
CON	5.63	6.47	4.55	6.42	Trt: NS
VITE	4.87	6.71	5.35	5.66	Stor*
SEM[e]	0.304	0.268	0.388	0.329	TrtxStor*
Tenderness					
CON	7.91	8.14	7.84	8.71	Trt: NS
VITE	8.37	8.56	8.33	8.20	Stor: NS
SEM	0.257	0.209	0.265	0.337	TrtxStor NS
Pork-flavor intensity					
CON	7.43	7.32	7.18	7.36	Trt: NS
VITE	7.64	7.55	7.28	7.01	Stor: NS
SEM	0.176	0.130	0.194	0.207	TrtxStor NS
Off-flavor intensity					
CON	14.45	14.26	13.83	13.99	Trt: NS
VITE	14.83	14.55	14.03	13.91	Stor*
SEM	0.157	0.116	0.162	0.208	TrtxStor NS

[a] No statistical differences were observed for 0 days and 7 days comparisons.
[b] Sensory measurements using a 15 cm line scale; 0 cm = extremely dry, tough, bland and intense off-flavor; and 15 cm = extremely juicy, tender, intense pork-flavor and no off-flavor.
[c] CON = control diet; VITE = diet supplemented with vitamin E.
[d] Repeated measures model effects: Trt = treatment, Stor = storage period, TrtxStor = treatment by storage interaction; * = $P < 0.05$, NS = not significant.
[e] Standard error of least squares means for storage within treatment.

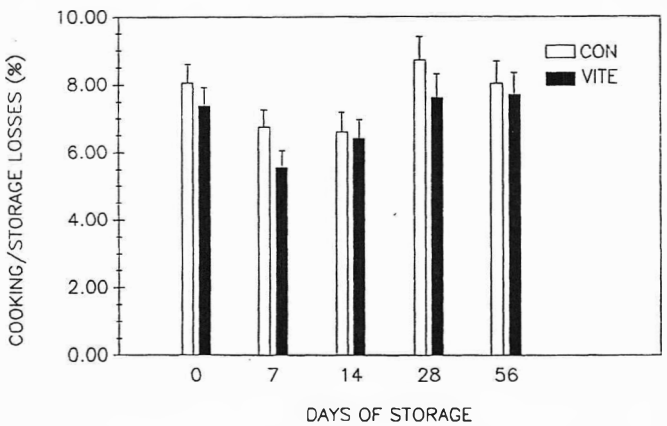

Fig. 2—Cooking/storage losses for precooked chops from controls and pigs supplemented with vitamin E. The following model effects were observed: Treatment = NS, Storage = * and Treatment × Storage interaction = NS; where * = $P < 0.05$ and NS = not significant.

familiarize them with changes that occur in pork during storage, specifically development of warmed-over flavor (WOF). Panelists used a 15 cm line scale with anchors and a midpoint (0 cm = extremely dry, tough, bland and intense off-flavor; 15 cm = extremely juicy, tender, intense pork-flavor and no off-flavor). Samples (70°C) were served with water (25°C) to members of the taste panel in a room where red lighting was used. Only five chops from each treatment were evaluated at 0 days compared to 15 chops/treatment evaluated at other sampling times. Reheating losses were determined by weighing samples before and after reheating. At 0 days, the same five chops from each treatment group used for sensory analysis were evaluated for reheating loss.

Proximate analysis was conducted on longissimus muscle from the chops used for the zero day evaluation. Closely-trimmed longissimus muscle samples were prepared by homogenizing in a food blender. Duplicate 3-g samples were used to evaluate moisture and lipid content using an oven drying procedure (70°C for 12 hr in a vacuum oven) and repetitive washes of petroleum ether in a Soxhlet extraction apparatus (AOAC, 1990).

Precooked roast study

At 4 days postmortem, closely trimmed semimembranosus/adductor muscles were removed from both fresh hams (n = 60) of each carcass and were used to represent a product prepared as a roast. Roasts were

Table 2—Total plate count (log CFU/g) of precooked chops and roasts from controls and pigs supplemented with vitamin E

Trait and treatment[a]	Days 0	7	14	28	56	Model effects[b]
Precooked chops						
CON	2.01	3.12	1.91	2.77	3.00	Trt NS
VITE	2.14	3.08	1.97	2.66	3.02	Stor*
SEM[c]	0.081	0.080	0.039	0.257	0.013	TrtxStor NS
Precooked roasts						
CON	1.91[x]	3.00	1.96[x]	1.94	3.00	Trt*
VITE	2.57[y]	3.00	2.43[y]	1.88	3.00	Stor*
SEM	0.122	0.122	0.122	0.122	0.122	TrtxStor*

[a] CON = control diet; VITE = diet supplemented with vitamin E.
[b] Repeated measures model effects: Trt = treatment, Stor = storage period, TrtxStor = treatment by storage interaction; * = $P < 0.05$, NS = not significant.
[c] Standard error of least squares means for storage within treatment.
[x,y] Means in the same column within each trait lacking a common superscript letter differ ($P < 0.05$).

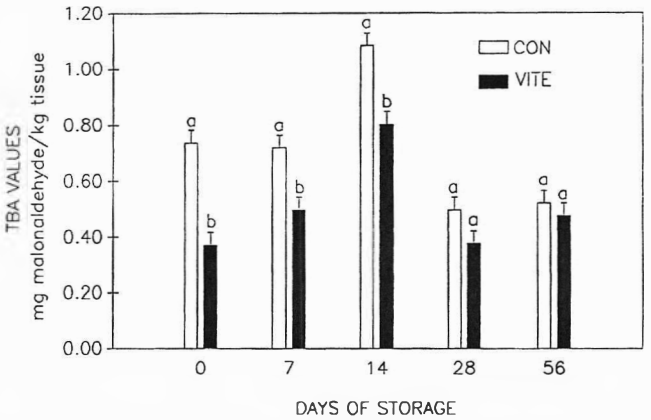

Fig. 3—TBA values for precooked roasts from controls and pigs supplemented with vitamin E. The following model effects were observed: Treatment = *, Storage = * and Treatment × Storage interaction = *; where * = $P < 0.05$ and NS = not significant. [a,b]Treatment means within the same storage period lacking a common superscript letter differ ($P < 0.05$).

vacuum packaged (−0.8 bar) in cook-in-bags (CN-530, Cryovac Division, W.R. Grace & Company, Ft. Worth, TX). Packaged roasts were steam-cooked to internal temperature 60°C and showered (21°C) for 10 min. Roasts were randomly assigned, by treatment, to five storage times of 0, 7, 14, 28, or 56 days. Roasts were held at 2°C for the specified storage period. Roasts assigned to the 0 days storage period were evaluated after a 24 hr cooling period. Sensory evaluation, TBA analysis, TPC, pH, storage losses and reheating losses were determined at the end of each storage period.

Cooking/storage losses were determined from weights recorded prior to packaging and immediately after removal of roasts from opened packages. Cooking/storage losses were not calculated for the product evaluated at 0 days. To provide enough pork for the entire experiment, roasts were cut into 2.54 cm slices at specified storage periods, and TBA, TPC and pH measurements were determined using procedures described for the precooked chop study. Tissue pH values were not obtained for samples stored for 7 days.

Taste panel slices were reheated in a Hobart Model DN 97-19 convection oven (Hobart Corporation, Troy, OH) at 149°C to internal temperature 70°C. Sensory evaluation was conducted employing procedures used for precooked chops. Reheating losses were determined by weighing slices prior to and immediately after heating.

Proximate analyses were conducted on duplicate 3-g samples taken from one precooked roast from each carcass. Moisture and lipid contents were determined using the same procedures as for the precooked loin chop study.

Statistical analysis

Individual taste panel scores were averaged across panelists using least squares means. All data were analyzed using the General Linear Model procedures of SAS Institute, Inc. (1986). For the precooked chop study, TBA, TPC, pH, cooking/storage losses, reheating losses and taste panel

Table 3—Sensory properties of precooked roasts from controls and pigs supplemented with vitamin E

Trait[a] and treatment[b]	Days 0	7	14	28	56	Model effects[c]
Juiciness						Trt NS
CON	8.34	8.81	7.12	3.92	6.83	Stor*
VITE	8.34	9.53	7.85	4.29	6.20	TrtxStor NS
						SEM[d] = 0.514
Tenderness						Trt*
CON	7.83[x]	8.42[x]	7.71[x]	7.64	8.53	Stor P = 0.08
VITE	9.00[y]	9.73[y]	8.97[y]	8.34	7.94	TrtxStor P = 0.09
						SEM = 0.386
Pork-flavor intensity						Trt NS
CON	7.14	7.41	7.50	7.85	7.29	Stor NS
VITE	7.28	7.46	7.54	7.74	7.44	TrtxStor NS
						SEM = 0.272
Off-flavor intensity						Trt*
CON	14.26	13.56	14.33	13.24	13.77	Stor*
VITE	14.86	14.25	14.49	14.00	14.40	TrtxStor NS
						SEM = 0.278

[a] Sensory measurements using a 15 cm line scale; 0 cm = extremely dry, tough, bland, intense off-flavor and unpalatable; and 15 cm = extremely juicy, tender, intense pork-flavor, no off-flavor and palatable.

[b] CON = control diet; VITE = diet supplemented with vitamin E.

[c] Model effects: Trt = treatment, Stor = storage period, TrtxStor = treatment by storage interaction; * = $P < 0.05$, NS = not significant.

[d] Standard error of least squares means for treatment x storage effects.

[x,y] Means in the same column for each trait lacking a common superscript letter differ ($P < 0.05$).

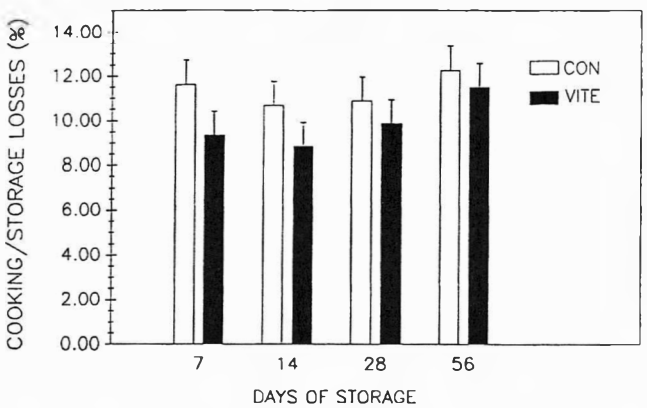

Fig. 4—Cooking/storage losses for precooked roasts from controls and pigs supplemented with vitamin E. The following model effects were observed: Treatment = P = 0.05, Storage = NS and Treatment × Storage interaction = NS; where * = $P < 0.05$ and NS = not significant.

data were analyzed using a repeated measures model that included the fixed effect of treatment and storage period as a repeated measure. Because only 10 chops were evaluated for sensory characteristics and reheating loss at zero days storage, two repeated measures analyses were conducted. One analysis compared chops used at 0 days to those same chops at 7 days and the other compared sensory evaluations and reheating losses of chops determined at 7, 14, 28 and 56 days storage.

Data for precooked roasts (TBA, TPC, pH, reheating losses, storage losses and taste panel evaluations) were analyzed using a completely randomized design. The model included the fixed effects of treatment and storage period, and interactions between the two effects. Lipid and moisture data for the chop study and roast study were analyzed using a complete randomized block design. The block design utilized the pen blocks of the feeding period (Cannon et al., 1995) to account for weight variation in the pigs.

RESULTS & DISCUSSION

THE MOST IMPORTANT TRAIT affecting acceptability and, thus, marketability of precooked pork products is the presence/absence of rancid flavor (WOF) associated with lipid oxidation (Tims and Watts, 1958). The processing and ingredients used to manufacture precooked pork products are critical in minimizing lipid oxidation. Supplementing pigs with vitamin E during the finishing period yielded pork that was less susceptible—as fresh and cooked product—to lipid oxidation during storage (Monahan et al., 1990a,b; 1992b). In our previous studies (Cannon et al., 1995), α-tocopherol was 10-fold higher ($P < 0.05$) in longissimus muscle from pigs supplemented with vitamin E (1.86 ± 0.20 μg/g tissue) than in that from pigs on a control diet (0.19 ± 0.03 μg/g tissue). From those results, we concluded that vitamin E was effectively incorporated into muscle through supplementation in growing and finishing diets.

Precooked chop study

Percentage moisture and percentage lipid as well as pH were not different ($P > 0.05$) for precooked chops from pigs supplemented with vitamin E as compared to those from pigs fed controls (data not presented in tabular form). Lipid oxidation, by TBA values, was consistently lower ($P < 0.05$) for VITE chops than for CON chops (Fig. 1). Lower TBA values for cooked chops from pigs fed supplemental vitamin E agreed with Monahan et al. (1990a,b) who stored cooked chops for times shorter than ours. A significant storage effect and a significant storage by treatment interaction on TBA values were reported. Lipid oxidation peaked after 14 days storage and there was a decrease, consistent for both treatment groups, in TBA values in chops stored for 28 days vs 14 days. The TBA values were below the threshold value (1.0 mg malonaldehyde/kg tissue) for detection of WOF (Boles and Parrish, 1990). Gray and Pearson (1987), summarizing previous research (Tarladgis et al., 1960; Greene and Cumuze, 1982), noted that rancid flavor was initially detected between TBA 0.5 and 2.0. The relatively low extent of lipid oxidation could be attributed to the cook-in-bag process, which removed oxygen by vacuum packaging prior to cooking. Previous research has also supported the use of vacuum packaging as a means of reducing lipid oxidation in precooked pork (Jones et al., 1987; Boles and Parrish, 1990).

Sensory characteristics of precooked chops from control pigs and those supplemented with vitamin E were not significantly different during storage (Table 1). Significant storage (7 days through 56 days) effects existed for juiciness and off-flavor intensity, and treatment by storage interaction was significant for juiciness. All values for tenderness, pork-flavor intensity and off-flavor intensity fell within an acceptable range (we assumed that sensory values > 7.5 were acceptable). Our findings indicated that under these processing and storage conditions, precooked chops could be successfully stored for ≥ 56 days.

Cooking losses/storage losses were not different ($P > 0.05$) for VITE chops and CON chops throughout storage (Fig. 2). Time of storage had a significant effect on weight losses; however, no consistent trend was observed over duration of storage. Reheating losses were not different ($P > 0.05$) between the two treatment groups at different storage times (data not presented in tabular form).

No differences ($P > 0.05$) in TPC were found between treatments at any given storage time, but during the storage period, counts increased ($P < 0.05$) by ≈ one log (Table 2). According to Ayres (1955), typical spoilage occurs at bacterial levels $10^7 \leq 10^8$ CFU/g. The TPC values we observed throughout storage were far below 10^7 indicating that, by cook-in-bag processing, precooked longissimus chops could be stored for ≥ 56 days.

Precooked roast study

Percentage moisture was lower ($P < 0.05$) in muscles of VITE roasts compared to that in CON roasts while lipid levels were similar in the roasts from the two treatments (data not presented in tabular form). Although the difference in percentage moisture was significant, the magnitude of the difference (73.57% compared to 72.63%) was very small. Treatment pH values were not different ($P > 0.05$), and pH changes over storage were minimal (data not presented in tabular form).

Over the entire storage, TBA values were consistently lower ($P < 0.05$) for VITE roasts than for CON roasts (Fig. 3). The magnitude of these differences was greatest at 0 days, 7 days, and 14 days storage. A storage effect and treatment by storage interaction were also observed ($P < 0.05$). The trends in lipid oxidation in the precooked roast study were similar to those in the precooked chop study. Only CON roasts stored 14 days had TBA values above the threshold for detection of WOF. These results indicate that precooking under vacuum and then storing under vacuum could minimize lipid oxidation over an extended period of time and that supplementation of vitamin E to the live animal could be used to further assure reduced lipid oxidation. The results revealing relatively low TBA values for the entire storage period in both treatment groups were similar to those by Jones et al. (1987) and Boles and Parrish (1990) who attributed limited lipid oxidation during extended storage to vacuum packaging prior to precooking.

Sensory characteristics of precooked roasts from pigs fed CON or VITE diets were compared (Table 3). Off-flavor intensity scores, which indicate degree of WOF, were consistently lower ($P < 0.05$) for VITE roasts than for CON roasts. A storage effect was found for off-flavor intensity ($P < 0.05$). Differences existed in taste-panel tenderness scores between treatments ($P < 0.05$). However, no previous research on feeding supplemental vitamin E to pigs has indicated differences in tenderness. A significant storage effect was observed for juiciness which tended to decrease as storage time increased. Juiciness scores were lowest for roasts stored 28 days. The magnitude of differences between VITE and CON roasts for off-flavor intensity scores as well as the acceptability level of these values (acceptable sensory scores > 7.5) reflect the low TBA values. These results indicate that precooked roasts, prepared and stored under such conditions, have acceptable sensory characteristics after storage for ≥ 56 days, and that adding supplemental vitamin E to the swine diet would help insure minimal detection of off-flavors.

Cooking/storage losses were consistently lower ($P = 0.05$) for VITE roasts than for CON roasts (Fig. 4). Reheating losses were not different ($P > 0.05$) between the two groups (data not presented in tabular form). Previous investigators have reported that vitamin E supplementation of swine diets significantly lowered storage drip-loss of fresh pork chops (Asghar et al., 1991; Monahan et al., 1992a). Buckley and Morrissey (1992) speculated that α-tocopherol molecules interacted with molecules in the cell membrane lipid bilayer and influenced the fluidity and integrity of the membrane. We could not conclude whether biochemical mechanisms involved in reducing storage loss were the same for precooked pork as those for fresh pork.

Significant treatment and storage effects and interactions between them were observed for TPC values (Table 2), which were higher for VITE roasts than for CON roasts at 0 days and 14 days storage. However, no consistent storage effects were detected and maximum counts did not exceed 3.0 log CFU/g. As with precooked chops, TPC values for precooked roasts were well below TPC levels at which products are considered spoiled.

Overall, our results suggested that cook-in-bag technology could be used to store precooked pork chops and roasts for at least 56 days. During this storage period, lipid oxidation and microbial growth could be minimized and sensory characteristics could be maintained at acceptable levels. Supplementation of vitamin E in the swine diet during the growing/finishing period can help minimize lipid oxidation in precooked pork.

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Ms received 2/2/95; revised 7/16/95; accepted 7/22/95.

This work was supported in part by Hoffman-LaRoche, Inc. and by the Colorado Agricultural Experiment Station.

Sensory and Texture Quality of Dry-Cured Ham as Affected by Endogenous Cathepsin B Activity and Muscle Composition

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ABSTRACT

Hams were processed with standard cure adjuncts under two drying temperatures and analyzed after aging for 7 mo. Dried hams revealed that flavor and texture traits were affected by moisture and nonprotein nitrogen (NPN) content and that NPN was enhanced by greater cathepsin B activity, lower salt levels and higher temperature. Two major defects of dry-cured ham, mushy mouthfeel and surface white film, occurred in about 15% of samples. Most of these hams had abnormal NPN values and were shown to originate from meat with high enzyme activity and low protein content. We concluded that this type of meat is prone to uncontrolled proteolysis and related undesired traits, and to be a potential hazard in dry-cured hams cured with less salt.

Key Words: ham, dry-cured, cathepsin B, sensory, texture

INTRODUCTION

TO IMPART DISTINCTIVE FLAVOR and mouthfeel to dry-cured hams, intensive handling and lengthy processing are required, resulting in high costs of production. For reducing costs the following have been studied: needle tenderization (Kemp et al., 1986), mechanical tumbling (Ockerman and Organisciak, 1978; Marriott et al., 1984), skinning or deboning of legs before curing (Moore et al., 1992) and time-temperature optimization (Lin et al., 1990). Published studies were reviewed by Marriott et al. (1992). In order to enhance the flavor of hams resulting from fast processing (Kemp and Fox, 1985), Marriott et al. (1987) used bacterial inoculation. Also, muscle proteolytic enzymes were taken into account as factors capable of affecting the sensory quality of dried hams (Toldrà et al., 1991, 1994; Parreño et al., 1994).

The effects of proteolytic enzymes in flavor and texture development depend essentially on the following: (a) dry-cured hams contain high amounts of free amino acids (FAAs) and low-molecular-weight compounds generally referred to as nonprotein nitrogen (NPN) (McCain et al.,1968; Buscailhon et al., 1993); (b) most cathepsins and aminopeptidases are active throughout processing, even at the low a_w values (0.90–0.92) typical of dried hams (Toldrà et al., 1992; Sárraga et al., 1993); and (c) aged ham flavor as well as texture qualities benefit from FAAs and NPN in acceptable dried hams (Careri et al., 1993, Buscailhon et al., 1994). However, in Italian long-aged heavy hams, high residual cathepsin B activity is often associated with high NPN values and abnormal softness, with most proteolyzed samples rated as mushy and unacceptable (Parolari et al., 1994).

Based on published reports, the use of proteolytic enzymes in ham processing appears rather contradictory, since enzymes have appeared to either improve flavor and texture or to impair the same traits if breakpoint proteolysis levels are exceeded.

Our objective was to determine the sensory and texture quality of hams as affected by naturally occurring levels of cathepsin B in pork. Among the traits investigated, two major defects, mushy mouthfeel and white film on the cut surface, were monitored as most likely influenced by FAAs or low-molecular-weight peptides, i.e, those compounds reported most often in defective hams (Butz et al., 1974; Toldrá et al., 1990; Arnau et al., 1994).

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MATERIALS & METHODS

Curing

Bone-in, unskinned unfrozen hams, 120, 9–11 kg each, were purchased from North European suppliers and were dry-cured on the third day after slaughtering. A cure mixture (99.0% NaCl, 0.5% $NaNO_3$ and 0.5% black pepper) was applied at 5 kg cure/ 100 kg meat in two applications at 7-day intervals. After curing 21 days at 1–4°C, 75–90% RH, hams were hung for an additional 50 days in a resting room at 1–3°C, 60–80% RH, for salt equilibration. Following washing with lukewarm water, and drying at 20°C, hams were grouped in two equivalent subsets and each treatment group was randomly assigned to a drying room held at either 15°C, 70–80% RH, or 18°C, 70–80% RH. After 1 mo, all hams were allowed to age at the same temperature (17°C) for an additional 3 mo. Average weight loss was 27% at the end of aging.

Samples

Slice portions (50–100g) of the semimembranosus muscle were removed from green hams prior to cure application and immediately submitted to chemical analyses. Hams at the end of aging or dry-cured hams were sectioned perpendicular to the bone, at knee-level. Four adjacent 2-cm-thick cushion sections comprising essentially the muscle semimembranosus (SM), semitendinosus (ST) and biceps femoris (BF) were vacuum-packaged and cold-stored for visual score and for flavor, texture and chemical analyses. L*a*b* values were also recorded prior to packaging on the samples taken for visual assessment.

Sensory analysis and visual score

An eight-member experienced sensory panel was trained for four sessions held at 2-day intervals 2 wk before testing sessions. Panelists during training were presented with four sensory attributes selected in a previous free-choice profile study of the same product (Parolari, 1994). Attributes and scale extremes were defined as follows. Saltiness, with 1 = 'not salty', 9 = 'extremely salty'. Dry-cured flavor, with 1 = 'flavor of uncured, green pork', 9 = 'flavor of fully matured dry-cured ham'. Bitterness, with 1 = devoid, 9 = 'bitterness of extremely proteolyzed dry-cured pork'. Softness, with 1 = 'extremely tough', 9 = 'mushy'. To improve attribute understanding, the upper extreme of the scale (score 9) was anchored with the following standards, which were provided throughout training for panel maintenance. Saltiness: dry-cured pork, 7–8% salt, 58–60% moisture. Dry-cured flavor: dry-cured ham, 12–14 months of aging, 5–6% salt, 58–60% moisture. Bitterness and softness: the bitter and mushy perception of dry-cured ham with 5–6% salt and with NPN (nitrogen-soluble after treatment with trichloroacetic acid) >33% of total muscle nitrogen. At the end of training all panelists were able to recognize the four given descriptors and to use them consistently. Individual ratings for each attribute fell within ±1 from the average panel score. Testing of the 120 hams was performed in 20 sessions of six random samples, at 2- to 3-day intervals. Each panelist received two thin slices, or about 20g, defatted ham for each sample and was asked to rate the samples for the four given attributes in the following order: saltiness, dry-cured, bitterness, softness.

Color changes due to white film were assessed both visually and instrumentally on the samples after 15 days of cold storage. Panelists were asked to rate white discoloration on a 9-point scale with 1 'fully red, no white film' and 9 = 'thoroughly white, no redness'. Instrumental param-

Table 1—Composition[a] and instrumental data of 120 hams prior to and at the end of manufacturing

	Mean	Range	C.V.[b]
Green hams			
Moisture	74.0	70.2–76.8	1.8
Protein	22.4	19.6–25.3	5.5
Moisture/protein	3.30	2.81–3.81	6.9
pH	5.69	5.40–6.69	5.6
Drip-loss[c]	6.8	3.5–12.2	26.8
Cathepsin B[d]	1.73	0.73–4.15	41.5
Dry-cured hams			
Moisture	62.2	55.3–66.1	2.1
Protein	25.7	22.6–30.4	4.3
Moisture/protein	2.40	1.84–2.69	7.4
Cathepsin B[d]	1.00	0.75–2.07	40.0
NPN[e]	27.6	22.0–36.2	10.1
Hardness (SM)[f]	3.91[g]	2.22–9.01	44.0
Hardness (BF)[f]	1.71[h]	0.73–4.37	45.7
Hardness (ST)[f]	1.47[h]	0.64–4.65	56.8

[a] Composition data are in grams per 100g lean meat.
[b] Coefficient of variation.
[c] As grams of liquid released by 100g lean meat.
[d] As nmol β-naphthylamine released $min^{-1} \cdot g\ meat^{-1}$.
[e] As grams of TCA-soluble non-protein nitrogen/100g total nitrogen.
[f] Force (kg) to compress muscle cores to 60% original height.
[g,h] Hardness (SM) significantly greater ($P < 0.05$) than hardness (BF) and hardness (ST).

Table 2—Correlation coefficients for hardness[a] and chemical data of dry-cured hams

Item	Hardness[a] SM	BF	ST
Moisture	−0.60**	−0.72**	−0.81**
Moisture/protein	−0.51**	−0.61**	−0.68**
Salt	0.47**	0.59**	0.61**
Proteolysis	−0.21	−0.31*	−0.31*
Cathepsin B	−0.25	−0.30*	−0.28*

[a] Hardness is force (kg) to compress 1-cm cores of muscles semimembranosus (SM), biceps femoris (BF) and semitendinosus (ST) to 60% original height.
* $P < 0.05$ ** $P < 0.01$.

eters L*a*b* were also recorded and their absolute differences from values measured at time zero, i.e., just after sectioning, were expressed as δL*, δa* and δb*.

Texture analysis

Two cores were taken, across the muscle fiber direction, from each 1-cm-thick semimembranosus (SM), semitendinosus (ST) and biceps femoris (BF) dry-cured muscle, with the aid of a 2-cm-diameter coring tool. Hardness was measured as the force (kg) to compress, parallel to the muscle fiber direction, each core to 60% of its original height using a 50-kg load cell mounted on an Instron Model 1122 Universal Testing Machine. It was operated at a crosshead speed of 5 cm/min, chart speed of 20 cm/min, and load cell setting ranging from 5 to 20 kg (5 for BF and ST, 20 for SM).

Chemical analysis

Green ham samples of SM muscle were analyzed for drip-loss, moisture, protein and cathepsin B activity. Drip-loss was determined by suspending ≈10g of 1-cm-thick muscle in plastic netting within a sealed, pre-weighed plastic bag for 24 hr at 2°C. The weight of the drip in the bag was expressed as percentage of weight of SM prior to dripping. Moisture and protein were determined following standard AOAC methods (AOAC, 1990).

Cathepsin B was assayed using a commercial kit (Cathepsin-3 test, Safe Food, 46019 Viadana, Italy), designed for rapid assay of cathepsin B in pork. The quick procedure is based on the following basic steps: meat (10g) is extracted with 100 mL citrate buffer, pH 5.0, containing 0.5M sodium citrate, 1 mM Na_2EDTA and 0.2% (w/v) Triton X-100 in a home mixer such as a Braun Minipimer (Braun, Barcelona, Spain) operated at speed setting #4 for 2 min, at ambient temperature. The slurry was filtered through a Whatman #4 and 0.25 mL filtrate was transferred into a 1-cm-diameter colorimeter grade reaction tube containing 0.75 mL buffer, pH 5.8, made up of 0.1M MES (2-N-morpholine ethanesulfonic acid), 10^{-3}M Na_2EDTA and $2 \cdot 10^{-3}$M cysteine. Following mixing for 1 min, 0.03 mL substrate solution (0.5 mM N-α-CBZ-Arg-Arg-4-methoxy-β-naphthylamide acetate in dimethylsulfoxide) was added and the mixture was allowed to react for 15 min at 37°C. The reaction was stopped by addition of 0.1 mL stopping solution (1M HCl and 2% Triton X-100) and then 1 mL dyeing reagent containing 0.05% *o*-dianisidine (w/v) in water was added and the mixture allowed to rest for 10 min at room temperature. Finally, absorbance was read at 520 nm and cathepsin B activity in the meat was expressed as nmol of β-naphthylamine released $min^{-1} \cdot g\ muscle^{-1}$. Prior to meat assay the kit procedure was tested against a standard fluorimetric assay for cathepsin B (Barrett, 1980) in the analysis of 25 fresh SM muscles, resulting in a high correlation ($r = 0.94$, $P<0.001$). The oversimplified procedure available with the kit enabled a batch of 12 samples to be assayed for cathepsin B in about 1 hr.

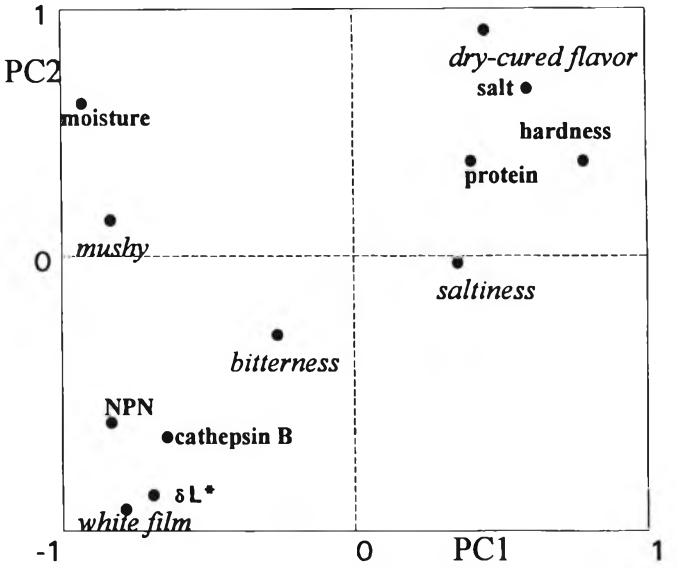

Fig. 1—Principal Components (PC) Analysis of chemical, texture and sensory data of dry-cured hams. Variance accounted for by axes: PC1 (35%), PC2 (25%). Sensory attributes are in italic. Hardness is the average of mechanical evaluations on muscle semimembranosus, semitendinosus and biceps femoris.

Trimmed lean (BF muscle) from dry-cured hams was analyzed for proximate composition and NPN. Moisture, protein and salt were determined by standard methods (AOAC, 1990). NPN, as grams of soluble nitrogen (after trichloroacetic acid addition)/100g total nitrogen in the meat, was obtained as previously reported (Careri et al., 1993). The substance removed by scraping from 20 sectioned hams exhibiting white film was analyzed for proximate composition (AOAC, 1990) and total free NH_2 compounds by the method of Moore and Stein (1954).

Data analysis

Correlation, regression, and principal components analyses were run by the SPSS-PC package (Norusis, 1986). Analysis of variance to compare means and χ^2 statistic to compare percent values were performed by the same package.

RESULTS & DISCUSSION

ALL HAMS APPEARED to have matured normally, according to conventional probe-and-sniff testing (Virgili and Parolari, 1991) and to weight losses, fitting the expected standards. Average chemical and instrumental data for green and dry-cured hams (Table 1) showed normal composition for uncured muscle compared with available data from proximate analysis of pork (Chizzolini et al., 1993). Drip-loss in fresh meat and cathepsin B in both fresh and dry-cured hams had large variation coefficients as did hardness values of dry-cured muscles. As expected, hardness of an external muscle such as semimembranosus was greater ($P<0.05$) than force to compress internal biceps femoris and semitendinosus.

Correlation coefficients (Table 2) show that mechanical texture was mainly related to aged muscle composition, with hardness substantially affected by ham shrinkage (moisture loss, salt

Table 3—Average composition of substance scraped from the cut surface of 20 hams exhibiting white film compared with standard dry-cured ham composition

Component	White film substance[a]	Dry-cured ham[b]
Moisture	51.7	60.4
Total nitrogen	26.7	26.0
Free amino groups[c]	34.1	11.0
Fat	15.4	4.6
Ash	6.1	7.5

[a] As grams component/100g scraped substance.
[b] As grams component/100g muscle. From Careri et al. (1993).
[c] Expressed as leucine. Grams/100 grams total nitrogen.

Table 4—Distribution of chemical properties and occurrence of defective dry-cured hams (mushy, white film) among quartiles[a] describing differing green leg characteristics

Quartiles	NPN[b]	Salt	Mushy[e]	White film[h]
I	26.8[c]	6.9	11[f]	18[i]
II	26.5[c]	6.4	15[f]	17[i]
III	28.0[c,d]	6.7	19[f]	16[i]
IV	28.7[d]	6.9	55[g]	48[j]

[a] Quartile distribution of multivariate scores obtained by the PC1 equation (as from Fig. 2): 0.91 × cathepsin B + 0.68 × moisture + 0.61 × drip-loss − 0.40 × pH − 0.89 × protein. According to the equation coefficients, quartiles I to IV describe legs with increasing enzyme activity, increasing moisture and decreasing protein content.
[b] Nonprotein nitrogen as percentage of total nitrogen in the meat.
[c-d,f-g,i-j] Different superscript letters denote significant difference.
[e] Per cent samples with oral softness score ≥ 7 (1 = extremely tough, 9 = extremely mushy). Different superscript letters denote significant difference by χ^2 statistic.
[h] Per cent samples with visual score ≥ 7 (1 = no white film, 9 = thoroughly white surface). Different superscript letters denote significant difference by χ^2 statistic.

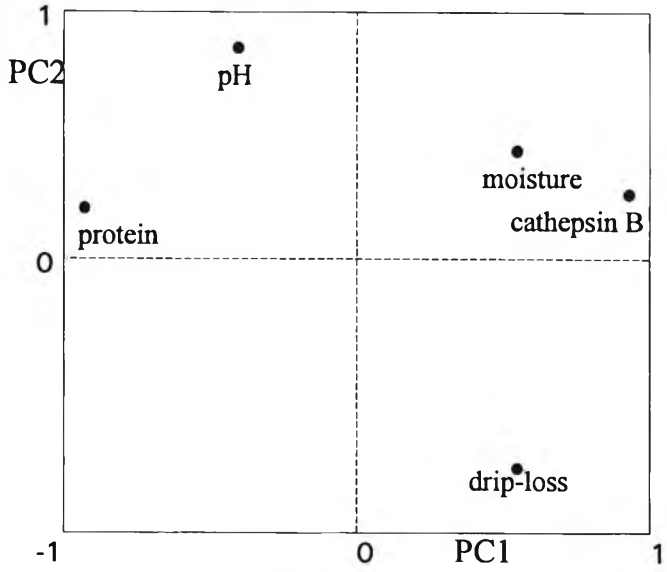

Fig. 2—Principal Components (PC) Analysis of chemical and instrumental data of green hams. Variance accounted for by axes: PC1 (45%), PC2 (20%).

increase) and, to a lesser extent, by nonprotein nitrogen (NPN) and cathepsin B activity. To better describe the ham properties in multi-variable terms, mechanical, chemical and flavor data were inspected by Principal Components Analysis (PCA), which indicated two factors or components accounted for 60% of original variance. The loading plot of variables under examination (Fig. 1) showed that texture hardness increased from left to right along the first axis or PC1 and was opposed to oral mushy texture. In agreement with previous correlation analysis (Table 2), hardness was enhanced by salt concentration and was contrasted by higher moisture content and nonprotein nitrogen. Moisture and NPN, positively correlated to the first axis, were split along the second component or PC2, suggesting that mushy samples were either high in moisture content or high in NPN values, with the high moisture samples 2:1 over the high NPN samples.

As shown by location on the PC1-PC2 plane, NPN and cathepsin B had very similar co-ordinates on both axes, indicating that proteolysis was essentially an enzyme-induced mechanism. Also, an undesirable trait and a major disadvantage to meat packers such as visual white film (described also by augmented instrumental brightness or δL^*) was closely linked to both NPN and cathepsin. This indicated that white discoloration was associated with enzyme-induced protein breakdown.

Several studies have included white film composition and possible linkage to storage conditions. Butz et al. (1974) attributed film development to tyrosine precipitation. An investigation on Spanish hams (Toldrà et al., 1990) reported low-weight-protein and free amino acids (FAAs) as prime chemical constituents of white film, suggesting that endogenous proteolytic enzymes were possible causes. Chemical analysis of white film scraped from our hams (Table 3) revealed many free amino groups, (as much as 34% of total nitrogen, compared with 11% in standard dried muscle). Abundance of NH_2 nitrogen in white film emphasizes the importance of enzyme-connected proteolysis in dried pork chemistry. Depending on enzyme activity in fresh meat, FAAs and, consequently, white film development will be enhanced or decreased in high- or low-activity muscle, respectively. This might explain the erratic occurrence of white film even on hams of the same batch or obtained under the same experimental conditions (Kemp et al., 1988). Being hydrophilic, most free amino acids or small peptides will eventually precipitate on low-moisture cut surfaces (white film and moisture had opposite co-ordinates on PC2, see Fig. 1). Furthermore, precipitation will be favored in a rather fatty medium as was found to be the scraped substance, containing in excess of 15% fat, compared with an average of 5% fat in dried muscle (Table 3).

The effects of processing were determined through the effect of salt, spanning over a 5.0–8.9% range in the 120 samples investigated, and through drying temperatures, held at treatment levels of 15°C and 18°C. In agreement with findings showing that cathepsin activity decreased on salt addition (Sárraga et al., 1989) NPN and salt had opposite loadings along the first principal component and, as a rule, white film did not form on hams with 7.5% NaCl or above. Moreover, NPN was higher in hams held at 18°C than in their counterparts at 15°C (average NPN=28.2 at 18°C vs NPN=25.9 at 15°C, $P<0.05$), according to published data (Rico et al., 1991) reporting cathepsin B enhancement by temperature increase in the range 15–25°C.

The effects of salt and temperature substantiated that enzyme-linked protein breakdown was a mechanism of tenderization of dry-cured ham. Defective mushy mouthfeel and white film result as a consequence of abnormal protein cleavage or uncontrolled proteinase activity. Note, however, that the proteolytic mechanism is also the prime source of those FAAs such as lysine or tyrosine that were found to contribute positively to distinctive flavor and the nutritional quality of matured ham (Careri et al., 1993). Thus the effects of protein cleavage on dry-cured ham quality look rather contradictory. This was expected for a proteinase-induced reaction, since fresh meat texture was shown to benefit from tenderizing calcium-dependent calpain activity (Goll et al., 1983; Koohmaraie et al., 1988), but injection of another proteinase such as papain into pig legs (Rogers et al., 1965) and beef roasts (McKeith et al., 1994) resulted in detrimental effects on mouthfeel. Unlike calcium-dependent proteinases, cathepsins have been demonstrated to remain active throughout the ham life span (Toldrà et al., 1991) and results from our study emphasize the stability of cathepsin B, which exhibited 40–60% retained activity even 7 mo after salting.

Our results demonstrated that cathepsin B was a factor capable of affecting proteolysis and related characteristics and that knowledge of enzyme levels in fresh meat would be helpful to ham manufacturers. It is to be stressed, in this respect, that cathepsin B exhibited wide fluctuations in green hams (Table 1) and was practically unrelated to instrumental measurements such

as pH or drip-loss that are currently run for quality control of fresh meat. Therefore, prediction of enzyme activity from available instrumental measurements and with sampling techniques commonly used in the ham industry would be difficult. Nevertheless, the green meat variables of the 120 hams, when analyzed by Principal Components Analysis, revealed a rather clear pattern for cathepsin B as linked with two major muscle constituents such as protein and moisture content. Figure 2 displays the cathepsin as opposed to protein on the more important PC1 (45% explained variance), whereas moisture and drip-loss were less strictly related to the same axis. The second, less meaningful factor (20% variance) showed an expected inverse relationship between pH and drip-loss. Note that those samples having highest scores along PC1, i.e., high-cathepsin, low-protein and high-moisture hams, were either mushy or presented white film after aging (Table 4).

CONCLUSION

LOW PROTEIN and, to a lesser extent, high moisture are muscle properties capable of enhancing the softening action of cathepsin B during maturation. Twelve of 120 or 10% of samples were simultaneously high in cathepsin B (above 1 standard deviation) and low in protein (below 1 s.d.), suggesting that a type of meat more liable to proteolysis may occur within meat batches normally supplied to ham processors and rated as regular by current measurements. This type of meat, or high proteolytic potential meat, is probably less suitable for processing into long-aged hams, particularly if low-salt processing is used to meet consumer demands. Checking raw meat for cathepsin B activity might be useful for preventing excessive proteolysis and related phenomena.

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Ms received 1/31/95; revised 4/24/95; accepted 5/22/95.

Formation of Biogenic Amines during Ripening of Dry Sausages as Affected by Starter Culture and Thawing Time of Raw Materials

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ABSTRACT

Raw materials affect formation of biogenic amines in dry sausages. Effects of thawing time of raw materials and amine-negative starter culture on amine formation were studied on a pilot scale. The levels of biogenic amines, precursor amino acids, pH, water activity, and microbial counts were measured. Use of starter culture significantly decreased levels of histamine, tyramine and cadaverine formed. The effect of thawing time on formation of biogenic amines was dependent on the use of starter culture.

Key Words: dry sausages, biogenic amines, thawing time, starter culture

INTRODUCTION

BIOGENIC AMINES are basic nitrogenous compounds occurring in foods due to decarboxylation of amino acids or transamination of aldehydes and ketones (Askar and Treptow, 1986). Some aromatic and heterocyclic amines, such as tyramine, histamine, tryptamine, and phenylethylamine, have vasoactive properties making their presence in food a potential public health concern. Food poisoning may occur especially in conjunction with potentiating factors such as amine oxidase-inhibiting drugs, alcohol, other food amines and gastrointestinal diseases. The aliphatic diamines putrescine and cadaverine, as well as the polyamines spermine and spermidine may also serve as indicators of spoilage (Askar and Treptow, 1986; Sayem-El-Daher et al., 1984; Taylor, 1990).

There are several reports of high levels of biogenic amines in fermented sausages from retail markets (Rice et al., 1975; Vandekerckhove, 1977; Pechanek et al., 1983; Pfannhauser and Pechanek, 1984; Bauer et al., 1989; Tschabrun et al., 1990; Vidal-Carou et al., 1990). On the basis of studies of retail dry sausages the differences in amine levels between different product types as well as between manufacturers could be a result of different processing environments and bacteria present (Vandekerckhove, 1977; Taylor et al., 1978; Rautiainen, 1988).

The production of biogenic amines in foods requires the presence of microorganisms capable of producing decarboxylase and a suitable cofactor and/or inducer for decarboxylation. Availability of an adequate concentration of amino acids, environmental factors favoring bacterial growth and conditions conducive to decarboxylase synthesis and subsequent decarboxylation are also required (Ferencik, 1970; Arnold and Brown, 1978; Edwards and Sandine, 1981; Kranner et al., 1991).

In order to avoid formation of hazardous levels of biogenic amines during ripening of dry sausages the critical factors for their formation must be known. Tschabrun et al. (1990) detected lower levels of histamine in dry sausages from fresh meat. Raw materials appear to be one of the main factors affecting amine formation in dry sausages (Kranner et al., 1991; Maijala, 1994). Biogenic amines are not formed in sterile meat (Slemr, 1981) and their levels increase in conjunction with microbial spoilage (Sayem-El-Daher et al., 1984; Schmitt and Schmidt-Lorenz, 1992), it can therefore be suggested that their formation in dry sausages is due to microbial activity. Different thawing times of deep-frozen raw materials are used by different sausage manufacturers. It is essential to know whether this thawing time affects microbial flora, the amounts of free amino acids as precursors and thus the formation of biogenic amines.

The addition of lactic acid bacteria cultures has been suggested to prevent histamine and tyramine accumulation by control of natural fermentation (Eitenmiller et al., 1978; Taylor et al., 1978). However, in the study of Rice and Koehler (1976) the use of *Pediococcus cerevisiae* and *Lactobacillus plantarum* did not result in lower levels of tyramine than when no starter culture was used. Furthermore in the study of Bauer et al. (1994) the addition of starter culture did not affect formation of biogenic amines. Starter cultures are almost always used in commercial dry sausage manufacture and new strains and combinations are continuously being developed. Therefore it is essential to know whether such strains have beneficial effects on formation of amines in dry sausages.

Our objective was to study the effects of thawing time of raw materials and addition of a histamine- and tyramine-negative starter culture on formation of biogenic amines during ripening of dry sausages.

MATERIALS & METHODS

Sausage manufacture

Sausages were prepared from deep-frozen (at −20°C for 1 wk) meat (38% pork, 26% pork fat, 32% beef). The raw materials were divided into three portions. Pork and beef meat were taken from a freezer (−18°C) to the refrigerator room (5°C) three (3d), two (2d) or one (1d) day before processing. Pork fat was taken to the refrigerator room 16 hr before processing to avoid technological problems. Each portion was further divided into two samples, of which one was fermented by the starter culture RM 2000 (Rudolf Müller and Co., Germany, containing *Pediococcus pentosaceus* and *Staphylococcus carnosus*) (S-sausages) and the other without starter culture (O-sausages).

The raw materials of each combination were chopped in a Seydelman cutter (K41) and NaCl (3%), $NaNO_2$ (120 mg/kg NO_2) as a 10% aqueous solution, glucose (0.6%), maltodextrose (0.1%), ascorbic acid (0.025%) and spices (0.5%) were added. Starter culture (in 0.1% peptone 0.85% NaCl water) was added to S-sausages to give $\log_{10}$ 6.6 staphylococci/g an $\log_{10}$ 7.3 pediococci/g. The chopping was started from the coldest raw materials, giving the following manufacturing order and mass temperatures before stuffing: 1d-0 (−3.1°C), 1d-S (−3.2°C), 2d-0 (−2.6°C), 2d-S (+3.4°C), 3d-0 (+6.5°C) and 3d-S (+9.5°C). The cutter was cleaned and disinfected by ethanol (70%) between batches.

Each batch of sausage mass was further divided into 18 small sausages (weight 350–400g). The sausages were ripened for 2 days at 23°C (93% relative humidity, RH), 1 day at 21°C (85% RH) and then 4 days at 20°C (85% RH). The remainder of the ripening and storage for up to 5 wk was at 10°C (70% RH). The sausages were lightly smoked during days 1–4.

Sampling

Three small sausages from each sausage type were taken as samples on the 3rd, 7th, 21st and 35th days of ripening. Biogenic amines, free amino acids, microbial counts, pH and water activity (a_w) were measured.

Microbiological analyses

A 10-g sample was serially diluted with a diluent containing 0.1% peptone and 0.85% NaCl in sterile deionized water. Coliform bacteria

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Table 1—Results of microbiological studies during ripening of dry sausages (log 10 CFU/g)[a]

Day of fermentation	Staphylococci		Lactic acid bacteria		Enterococci		Haemolytic bacteria		Yeasts	
	O	S	O	S	O	S	O	S	O	S
Thawed for 1 day										
0	< 2	< 2	2.7	2.7	< 2	< 2	2.3	2.3	2.0	2.0
3	3.4	6.3	7.2	8.7	< 2	< 2	2.7	< 2	2.1	< 2
7	4.9	6.4	7.7	8.3	4.6	< 2	4.6	< 2	2.2	< 2
21	5.4	6.4	7.3	8.7	3.3	< 2	4.5	< 2	3.4	< 2
35	4.1	5.7	7.3	8.0	2.5	< 2	5.1	< 2	< 2	< 2
Thawed for 2 days										
0	3.0	3.0	3.4	3.4	< 2	< 2	< 2	< 2	2.2	2.2
3	4.8	6.5	7.6	8.7	5.2	< 2	< 2	< 2	< 2	< 2
7	5.5	6.6	7.9	8.5	5.1	< 2	4.6	< 2	3.3	< 2
21	5.3	6.0	7.6	8.2	4.7	< 2	4.3	< 2	2.5	< 2
35	5.1	5.5	7.0	7.9	4.8	< 2	4.6	< 2	2.7	< 2
Thawed for 3 days										
0	2.2	2.2	3.6	3.6	< 2	< 2	2.0	2.0	3.2	3.2
3	5.1	6.7	7.7	8.7	3.9	< 2	< 2	< 2	3.6	2.0
7	5.2	6.4	8.0	8.2	3.5	< 2	4.5	2.7	4.4	< 2
21	5.0	5.7	7.6	8.3	2.7	< 2	4.2	< 2	3.9	< 2
35	4.9	5.7	7.0	7.8	3.5	< 2	4.2	< 2	3.2	< 2

[a] Means of three samples, except sausages of 35 days, which are means of six samples. O = no starter culture, S = starter culture used.

were enumerated on Violet Red Bile Agar (VRB, Merck, ISO method no. 4832, 1991), enterococci on Slanetz-Bartley agar (SB, Merck, NCFA method no. 68, 1992), molds and yeasts on malt extract agar (Oxoid) with added chlortetracycline (100 mg/L) and chloramphenicol (100 mg/L) incubated at 22°C for 3 days (yeasts) and 5 days (molds), staphylococci and haemolytic bacteria on blood agar base (BBL) containing 5% defibrinated horse blood (incubated at 37°C for 48 hr) and lactic acid bacteria (LAB) on de Man, Rogosa and Sharpe agar with sorbic acid [MRS-S, LabM MRS with sorbic acid of Fluka (*Pharmacopoeia of Culture Media for Food Microbiology,* 1987) incubated at 20–22°C for 5 days anaerobically].

The number of histamine-producing bacteria was measured from the raw materials and from the final sausages at 5 wk. Samples of 5 × 1g, 5 ×0.1g, 5 × 0.01g, and 5 × 0.001g (5.555g in all) of the raw materials and sausages were examined by the most probable number (MPN) method. Histidine-containing broth (HL) according to Kranner and Schopf (1992) was used. The ingredients (25.0g L-histidine-HCl (BBL 11929), 15.0g Standard II Nutrient Broth (Merck 7884), 5.0g Lab-Lemco powder (Oxoid L29), 5.0g yeast extract (BBL 11929), 0.1g $MgSO_4$, 0.05g $MnSO_4$, 2.0g KH_2PO_4) were mixed in 1L of distilled water and autoclaved for 15 min at 121°C. pH was adjusted to 5.1 by HCl before autoclaving. The same kind of broth but without histidine (K-HL) was used in the control MPN series, with the same number of tubes as with HL. Two histamine-positive lactic acid bacterial strains (G-106 and G-230), one histamine-negative lactic acid bacterium (GS-11) as well as histamine-negative *Escherichia coli* (NVIF-085) and *Klebsiella pneumoniae* (NVIF-030) were used as control strains for HL and K-HL. The positive tubes of both HL and K-HL were confirmed by spreading with a loop on EMB, S-B, blood and MRS-S agar plates. From these plates 3 pure cultures were further tested in HL and K-HL. If more gas was formed in HL than in K-HL the strains were regarded as positive for histamine production. If positive strains were detected the original MPN tube was counted as positive.

pH and water activity

pH values were measured directly from samples using a WTW pH 537 meter (Germany) equipped with an Ingold DXK-S7/25 electrode. After an equilibration period of 2 hr, a_w values were obtained at 25°C from a sample of 25–30g using a Rotronic Hygroskop (Fattore Vitale & Co. Italy).

Chemical analyses

Biogenic amines were extracted from 2g of sample with 0.4M perchloric acid and detected as their dansyl derivatives by high performance liquid chromatography (Eerola et al., 1993). The limits of determination for the amines were: tryptamine, histamine, cadaverine, spermidine and spermine 1 mg/kg, tyramine 2 mg/kg, putrescine 5 mg/kg and phenylethylamine 10 mg/kg. The precision values of the method calculated as CV% after extracting and analyzing the same sample six times were: tyramine, histamine and tryptamine 4%, spermine 5%, phenylethylamine 6%, cadaverine 7%, spermidine 9% and putrescine 18%. Precursor amino acids for amines (Guggenheim, 1951; Askar and Treptow, 1986): free glutamic acid and arginine (putrescine), histidine (histamine), tyrosine (tyramine), phenylalanine (phenylethylamine) and lysine (cadaverine) were extracted from 5g of sample with 10% trichloroacetic acid. The extract was evaporated and dissolved in 0.2M sodium citrate buffer, pH 2.2 (Stockemer, 1982). Amino acids were analysed as their o-phthalaldehyde derivatives using an automated HPLC system (Hewlett Packard, AminoQuant Analyzer) and fluorescence detection (Blankenship et al., 1989). The precision values of the method (CV%) for different amino acids were: phenylalanine 8%, lysine 9%, glutamic acid and arginine 10%, tyrosine 11% and histidine 18%.

Statistics

The effects of thawing time of raw materials and of starter culture addition were analysed by multiple analyses of variance (ANOVA) using

Table 2—Levels of free amino acids during ripening of dry sausages (mg/kg)[a]

Day of fermentation	Tyrosine		Histidine		Lysine		Arginine		Glutamic acid		Phenylalanine	
	O	S	O	S	O	S	O	S	O	S	O	S
Thawed for 1 day												
0	30	30	790	790	50	50	1140	1140	150	150	20	20
7	130	120	430	670	400	370	1800	1660	420	570	150	260
35	180	180	320	500	590	560	1890	2090	730	1030	250	320
Thawed for 2 days												
0	20	20	700	700	90	90	1340	1340	170	170	20	20
7	90	80	290	170	460	380	1830	1670	520	620	170	150
35	160	140	190	160	950	740	2200	2060	1040	960	290	240
Thawed for 3 days												
0	30	30	530	530	120	120	1020	1020	190	190	20	20
7	100	90	460	400	490	190	1240	1160	490	580	150	150
35	150	140	420	300	970	430	1700	1410	960	940	290	250

[a] Means of three samples, except sausages of 35 days, which are means of six samples. O = no starter culture, S = starter culture used.

Table 3—Levels of biogenic amines during ripening of dry sausages (mg/kg)[a]

Day of fermentation	Tyramine		Histamine		Cadaverine		Putrescine		Spermidine		Spermine	
	O	S	O	S	O	S	O	S	O	S	O	S
Thawed for 1 day												
0	< 1	*	1	*	*	*	6	*	5	*	28	*
3	3	3	2	2	< 2	*	6	6	6	6	29	28
7	8	6	3	2	8	3	6	6	6	6	31	25
21	16	14	7	4	32	3	11	9	8	8	36	35
35	39	23	7	5	92	12	18	13	8	7	52	55
Thawed for 2 days												
0	< 1	*	2	*	< 2	*	6	*	6	*	26	*
3	6	3	2	1	< 2	< 2	7	6	7	6	27	26
7	28	5	2	3	7	5	8	7	7	7	27	27
21	60	10	3	4	30	14	13	13	8	9	32	35
35	87	18	8	3	76	23	21	19	8	7	50	50
Thawed for 3 days												
0	< 1	*	1	*	< 2	*	7	*	6	*	29	*
3	5	3	2	2	< 2	< 2	7	7	7	7	28	25
7	17	5	2	2	3	6	7	7	7	7	30	28
21	45	9	10	3	13	14	11	16	9	8	34	26
35	48	16	13	3	32	30	18	24	9	9	51	47

[a] Means of three samples, except sausages of 35 days, which are means of six samples. O = no starter culture; S = starter culture used; * = not detected.

the EXCEL 4.0 Analysis ToolPak on an ICL MikroMikko 4 m 326 SX/PC.

RESULTS & DISCUSSION

Effect of thawing time on raw materials

The number of yeasts and LAB and the concentrations of free lysine and glutamic acid were higher ($p<0.05$) in raw materials with longer thawing times (Tables 1 and 2). Raw materials thawed for 2 days contained the most free arginine ($p<0.01$). No significance differences occurred either in levels of biogenic amines (Table 3) or in pH (Fig. 1) and a_w (Fig. 2) of the raw materials as a function of thawing time.

Effect of starter culture during ripening

Addition of starter culture increased the levels of staphylococci and LAB ($p<0.001$) during ripening of dry sausages, as expected (Table 1). The numbers of enterococci, haemolytic bacteria and yeasts ($p<0.001$) as well as the levels of tyrosine ($p<0.01$) and lysine ($p<0.05$) were lower in sausages manufactured using starter culture (S-sausages) than in those manufactured without starter culture (O-sausages) (Tables 1 and 2). S-sausages also contained less tyramine, histamine and cadaverine ($p<0.001$) than O-sausages (Table 3).

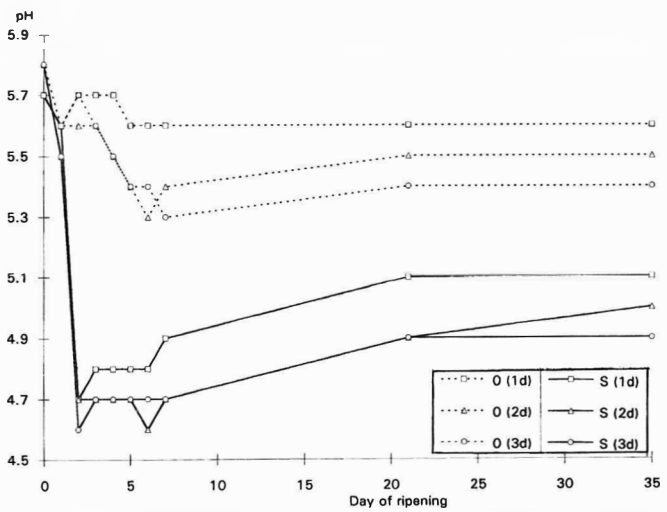

Fig. 1—pH-values during ripening of sausages. O = no starter culture added, S = fermented with starter culture. Raw material thawed for 1, 2 or 3 days (d).

pH decreased more rapidly and to lower levels in S-sausages than in O-sausages (Fig. 1). At the beginning of fermentation a_w-values were similar in all the sausage groups. However, at 5 wk the a_w-values were lowest in S-sausages manufactured from raw materials thawed for 1 and 2 days ($p<0.05$) (Fig. 2).

Effect of thawing time during ripening

During ripening of dry sausages the effect of thawing time of raw materials on numbers of bacteria detected was dependent on use of starter culture. In S-sausages thawing time had no effect on numbers of bacteria studied (Table 1). However, in O-sausages a longer thawing time of raw materials resulted in higher levels of yeasts ($p<0.05$). The number of enterococci was highest in O-sausages manufactured from raw materials thawed for 2 days ($p<0.05$).

The effect of thawing time of raw materials on formation of biogenic amines was also dependent on use of starter culture. In S-sausages the levels of tyramine ($p<0.001$) and spermine ($p<0.01$) were lower and levels of putrescine were higher ($p<0.001$) when the thawing time of raw materials was longer (Table 3). In O-sausages, however, the levels of histamine ($p<0.01$) increased as a function of thawing time. Interestingly, even when levels of lysine in raw materials increased with longer thawing time the levels of cadaverine (of which lysine is a precursor) decreased during ripening in O-sausages ($p<0.001$) and increased in S-sausages ($p<0.001$) as a function of the thawing time.

The thawing time affected levels of free amino acids during ripening of dry sausages regardless of use of starter culture. The levels of tyrosine ($p<0.001$) and arginine ($p<0.05$) decreased with longer thawing times of raw materials (Table 2). The greatest decrease and lowest levels of histidine ($p<0.01$) were observed in sausages from raw materials thawed for 2 days.

pH decreased to lower levels when thawing time of raw materials was longer ($p<0.001$) both in S- and in O-sausages (Fig. 1). a_w-values were higher ($p<0.05$) at the age of 3 and 5 wk of dry sausages (Fig. 2) when raw materials were thawed for 3 days.

The MPN values for histamine-producing bacteria in raw materials after thawing for 3 days, 2 days and 1 day were $\log_{10}$ 3.3, 2.3, and 2.3 CFU/g, respectively. However, no histamine-producing bacteria were detected in any of the sausages studied at an age of 5 wk. This conformed well with the low levels of histamine detected in the sausages.

No coliforms ($<\log_{10}$ 1.9 CFU/g), tryptamine or phenylethylamine (<10 mg/kg) were detected in any samples. Neither thawing time nor starter culture had any effect on formation of spermidine.

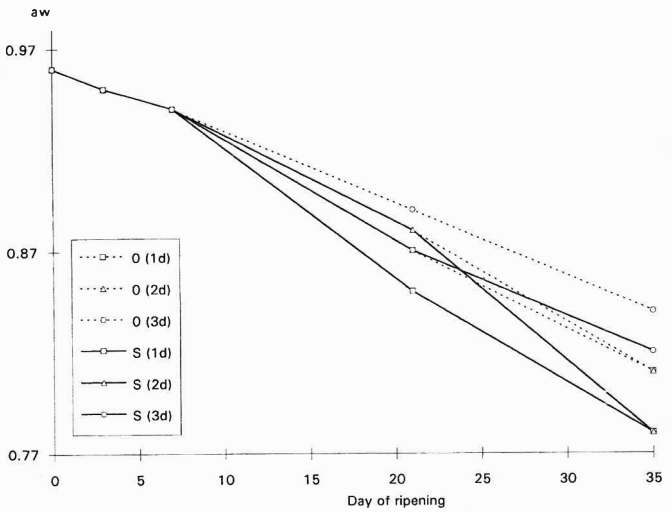

Fig. 2—a_w-values during ripening of sausages. O = no starter culture added, S = fermented with starter culture. Raw material thawed for 1, 2 or 3 days (d).

Discussion

In sausages manufactured with starter culture, the levels of tyramine and cadaverine as well as their precursors tyrosine and lysine were lower than in sausages manufactured without starter culture. Levels of histamine were also lower, although there were no differences in levels of histidine. Therefore these results support the use of starter culture to decrease formation of biogenic amines. However, note that there are great differences between starter strains on the market. The different reported results of beneficial effects of starter cultures (e.g. Eitenmiller et al., 1978; Taylor et al., 1978; Rice and Koehler, 1976; Bauer et al., 1994) may be a consequence of differences between strains or sausage types or possibly unsuitable processing programs may have been used for some starter cultures. As was also seen in our results, the formation of biogenic amines in dry sausages was affected by many different factors which may have opposing effects. For example, levels of cadaverine decreased in O-sausages but increased in S-sausages with longer thawing times of raw materials.

Bauer et al. (1994) indicated that tyramine represents a suitable indicator for long-stored meat, or for the fact that long-stored, even spoiled meat, has been used in production of fermented sausages. However, in our study levels of tyramine, histamine and cadaverine of dry sausages were decreased when starter culture was used. These results suggested that biogenic amines cannot be directly applied as a quality parameter for raw materials for dry sausages, in agreement with results of Vidal-Carou et al. (1990) and Maijala et al. (1995). The same raw materials can lead to very different amine levels in final products depending on factors such as the starter culture or fermentation temperature used. For example, it is possible to obtain sausages with high levels of tyramine from high quality raw materials with an incorrect processing program and/or starter culture. However, high levels of biogenic amines in the retail dry sausages seem to reflect problems either in raw materials or in the formulation, starter culture or processing program (Maijala, 1994).

The use of starter culture as well as thawing time of raw materials affected formation of biogenic amines in dry sausages. These effects were mutually dependent. The starter culture used had a beneficial effect by decreasing formation of biogenic amines. The ultimate effect of thawing time was dependent on use of starter culture. However, we concluded that a thawing time of raw materials up to 3 days at 5°C could be used for dry sausage manufacture if a starter culture and good quality raw materials are used.

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Ms received 3/29/95; revised 6/15/95; accepted 6/24/95.

This study was supported by the Food Research Foundation, Finland, and the Academy of Finland.

We thank Kirsi Lahti for technical assistance in the laboratory.

Quaternary Ammonium Compounds Inhibit and Reduce the Attachment of Viable *Salmonella typhimurium* to Poultry Tissues

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ABSTRACT

Quaternary ammonium compounds (QAC) were studied to characterize their inhibition and reversal of *S. typhimurium's* strong attachment to chicken skin. An initial screening human buccal epithelial cell model and a novel, highly quantitative model using pieces of chicken skin in culture plates were used. Some QAC, particularly cetylpyridinium chloride (CPC), were extremely effective at both inhibiting and reversing attachment of viable *S. typhimurium* to chicken skin. Pretreatment of chicken skin (room temperature, 10 min) with 0.1% CPC completely inhibited the attachment of *S. typhimurium*. CPC may be very beneficial in reducing *S. typhimurium* contamination of poultry carcasses during processing and especially in preventing cross-contamination.

Key Words: poultry, salmonella, quaternary ammonium, skin-attachment, *S. typhimurium*

INTRODUCTION

PREVENTION OF FOOD BORNE ILLNESS by microbiological contamination is of major concern to the poultry and meat processing industry, regulatory agencies, and consumers. Pavia and Tauxe (1991) estimated that 2–4 million cases of salmonellosis may occur annually in the United States, and that patient care costs amounted to billions of dollars. *S. typhimurium* contamination is of special concern to the poultry processing industry because the organism is often present on live birds (Cunningham, 1982; Lahellec et al, 1986).

Poultry processors have encountered major difficulty in removing those microorganisms that become adherent, or attach rigorously, to poultry tissues. *S. typhimurium* can become very firmly attached to poultry and meat tissues. Those microorganisms that do not attach can be washed off easily, while those that become strongly attached cannot be removed by rinsing and become more resistant to removal by chemical or physical means. This strong attachment has been well studied, but chemical and biochemical mechanisms remain unclear (Firstenberg-Eden et al., 1978; Schwach et al., 1982; Soerjadi et al., 1982; Lillard, 1985, 1986, 1990; Kristiansen et al., 1987; Finlay et al., 1989).

A variety of chemical and physical approaches have been utilized during poultry processing to eliminate *S. typhimurium* contamination of carcasses and minimize cross-contamination among carcasses (Morrison and Fleet, 1985; Izat et al., 1989). However, some such processes may adversely affect appearance, color, flavor, and/or texture of the products. Also, existing technologies are not completely effective in removing all attached microorganisms from poultry and meat tissues (National Research Council, 1985, 1987). A more effective process for removal and prevention of *S. typhimurium* contamination as well as elucidation of mechanisms involved would be of high value to food processing industries.

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The adhesion (strong attachment) of microorganisms to poultry appears to be a surface interaction phenomenon (Firstenberg-Eden et al., 1978; Schwach et al., 1982; Soerjadi et al., 1982; Lillard, 1985, 1986, 1990, Kristiansen et al., 1987; Finlay et al., 1989). Investigation of the effects of surface-active agents (surfactants) on bacterial attachment could provide information regarding more effective agents for removal of attached organisms or for prevention of attachment, as well as chemical probes for studying the biochemical mechanism. Cationic surfactants such as alkylpyridinium salts might be very effective, inasmuch as carboxylate (negatively charged) functional groups are prevalent on the exterior surface of bacterial cell walls (Neihof and Echols, 1978). Russell and Furr (1987) reported the differential sensitivity of wild-type and mutant strains of *E. coli* to two quaternary ammonium compounds, benzalkonium chloride and cetylpyridinium chloride. They observed that deep rough (lipopolysaccharide-defective) mutant strains were much more sensitive than wild-type strains to quaternary ammonium compounds. Our preliminary investigations of surfactants effects on protein binding to *S. typhimurium* demonstrated that quaternary ammonium surfactants were much more effective than anionic or neutral surfactants, mannose, or protamine as inhibitors of ^{14}C-chicken egg albumin binding to *S. typhimurium*.

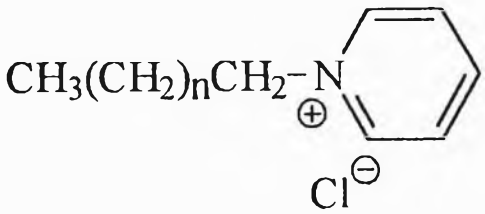
Our objective was to characterize the activity of a series of quaternary ammonium compounds (QAC) to inhibit and/or reduce attachment of viable *S. typhimurium* to poultry tissue. We utilized both human buccal epithelial cell and poultry skin laboratory models.

MATERIALS & METHODS

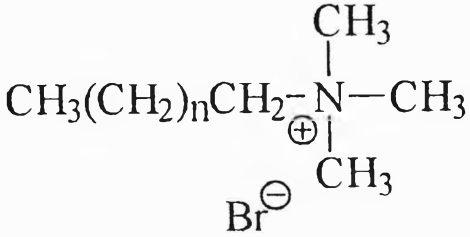
CHEMICAL COMPOUNDS were reagent grade, commercially available (Aldrich Chemical Company), and were used as purchased without further purification. Trisodium phosphate (TSP) was used as the dodecahydrate ($Na_3PO_4 \cdot 12\ H_2O$). Chemical structures and names of the QAC included in this study are shown in Fig. 1. All studies were conducted using *S. typhimurium* ATCC 14028. Prior to use, bacteria were grown on nutrient agar (BBL Microbiology System) for 16 hr at 37°C, suspended in 0.008M phosphate buffered saline (PBS, pH 7.2), washed twice at 4800 rpm for 10 min and diluted in PBS to obtain a final cell concentration (spectrophotometrically, 420 nm) of 5–10 × 10^8 cells/mL. All experiments were conducted at room temperature (≈25°C) unless otherwise indicated. Unprocessed chicken skins were obtained frozen from the Poultry Processing Pilot Plant at the University of Arkansas at Fayetteville and had not been subjected to any chemical or physical microbiological decontamination. Squares (2.5 × 2.5 cm) of chicken skin excised from drumsticks were used in reduction and inhibition experiments.

Bactericidal activity of QAC

Viability study. A 16-hr culture of *S. typhimurium* in nutrient broth was centrifuged (15,000 rpm, 10 min, 4°C), the supernatant was removed, the pellet was washed with 10'mL 0.04M potassium phosphate buffer (PPB, pH 7.0), and resuspended in PPB to provide a final suspension of 1–2 × 10^9 cells/mL. Aliquots (1.0 mL) were added to 1.8-mL Eppendorf vials, centrifuged (14,000 rpm, 4 min), and supernatants were removed. Each pellet was suspended in either 1 mL of an aqueous soln of various concentrations of test compound or 1.0 mL of PPB,



Cetylpyridinium chloride (CPC), n = 14
Dodecylpyridinium chloride (DPC), n = 10



Dodecyltrimethylammonium bromide (DTMAB), n = 10
Tetradecyltrimethylammonium bromide (TTMAB), n = 12
Hexadecyltrimethylammonium bromide (HTMAB), n = 14

Fig. 1—Structures and names of quaternary ammonium compounds (QAC).

mixed on a vortex (30 sec), and centrifuged (14,000 rpm, 4 min). The supernatant was removed, each pellet was washed with PPB (1 mL, 14,000 rpm, 4 min), the supernatant removed, and each pellet was re-suspended in 0.5 mL PPB. Cells from each sample were counted using duplicate 0.05-mL aliquots and standard serial dilution techniques on nutrient agar, and the data recorded as mean colony-forming units (CFU)/mL.

MIC study. Minimum inhibitory concentrations (MIC) for QAC against *S. typhimurium* were determined in Mueller Hinton broth (BBL Microbiology System) using the macrodilution method established by the National Committee for Clinical Laboratory Standards (1987). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were included in each assay as reference strains. The standard reference compound ampicillin was used to validate the process.

Effects of QAC on reduction of *S. typhimurium* on human buccal epithelial cells (HBE). The activity of QAC to reverse and inhibit the attachment of *S. typhimurium* to HBE cells was determined using a modification of the procedure described by Boussard et al. (1989).

Reversal of attachment. The HBE cells were obtained by swabbing the buccal surface of a healthy, nonsmoking donor. The cells were suspended in 10 mL PBS, washed (10 mL, 2X) at 4800 rpm for 10 min; the number of living cells was estimated, and the suspension was diluted (PBS) to a final concentration of 5–10 × 10^4 cells/mL. A suspension of the HBE cells was mixed with an equal volume of either the bacterial suspension (3.0 mL), or PBS (0.8 mL) as a background bacteria control. Each mixture was agitated gently (1.5 hr, 35°C), centrifuged (4800 rpm, 10 min), and 1 mL of each was transferred into Eppendorf vials followed by washing with PBS (1 mL, 1500 rpm, 10 min) to remove bacteria that had not become attached to HBE cells.

The HBE cells were treated with either 1.0 mL of an aqueous soln of various concentrations of the QAC or 1.0 mL PBS (treatment control) by mixing on a vortex at low speed (30 sec) followed by centrifugation (1500 rpm, 10 min). Following treatment the cells were washed once with PBS (1 mL), erythrosin B (20 µL) was added to each vial, the suspension was centrifuged (1500 rpm, 10 min), the cells were washed by centrifugation with PBS (1 mL, 2X), and resuspended in 300 µL PBS. The suspension was spread on microscope slides, dried overnight, and counterstained with methylene blue. The number of *S. typhimurium* remaining attached to HBE cells was determined by counting the organisms on 30–50 HBE cells from each suspension, subtracting the average number of background organisms determined on the uninoculated control cells, and calculating the mean number (± SD).

Inhibition of attachment. The HBE cells were prepared as described, and 1-mL aliquots of suspension were treated with either 1 mL of an aqueous soln of various concentrations of QAC or 1 mL of PBS (treatment control) followed by vortexing at low speed (30 sec) and centrifugation (1500 rpm, 10 min). The supernatant was withdrawn and the HBE cell pellet was then treated with either 1 mL of the *S. typhimurium* suspension (5–10 × 10^8 cells/mL) or 1 mL of PBS (inoculation control). All subsequent procedures were performed as described for reversal of attachment.

Effects of QAC on attachment of *S. typhimurium* to chicken skin

Reduction of viable *S. typhimurium*. Chicken skin (2.5 × 2.5 cm) excised from a drumstick was placed epidermal side up in each well of a 6-well tissue culture plate. One skin in each group of six was used as a background control and treated with 5 mL PBS; each remaining skin was inoculated with 5 mL PBS containing 6–8 × 10^3 CFU/mL *S. typhimurium*. The plate was incubated (30 min, 35°C), and each skin was rinsed (2X, 5 mL PBS) to remove loosely bound (unattached) organisms. A positive control skin was treated with 5 mL of PBS, remaining skins were treated with 5 mL of various concentrations of test compound in PBS, and the plate was incubated with shaking (100 rpm) for various times and at various temperatures. After incubation, skins were rinsed once (5 mL PBS), and a nitrocellulose lift was taken from both the upper and lower surfaces of each skin to recover bacteria on the surfaces.

The nitrocellulose lift was performed using a sterile glass spreader to press 1 nitrocellulose square (3 × 3 cm) to the upper surface (epidermal) of the skin and another square to the lower surface of the skin. The squares (two for each skin) were removed from the skin with sterile forceps, placed on a XLD agar plate, incubated (37°C, 18–24 hr), and the *S. typhimurium* colonies on each plate were counted. The recovery of *S. typhimurium* from each skin was expressed as CFU/skin. Each experiment was performed in triplicate.

An alternate method for recovery of *S. typhimurium* involved use of a laboratory blender (Stomacher 400, Seward Medical, London, England) to homogenize the skin. Each square of skin was placed in 80 mL of sterile saline and homogenized by use of the Stomacher for 2 min. This homogenate (1 mL) was pour-plated with XLD agar and incubated (37°C, 18–24 hr). *S. typhimurium* colonies were counted, corrected for dilution, and reported as CFU/skin. Each experiment was performed in triplicate.

Inhibition of attachment. Chicken skins were placed in 6-well culture plates as described for reduction of viable *S. typhimurium*. A PBS solution (5 mL) was added to the positive control skins, and 5 mL of solutions of various concentrations of test QAC or TSP was added to the remaining wells. The culture plates were incubated with shaking (100 rpm) for various times at various temperatures. The test solution was removed by aspiration, and skins were incubated (30 min, 35°C) with 5 mL of PBS containing 6–13 × 10^3 CFU/mL *S. typhimurium*. The incubating solution was removed by aspiration, and skins were rinsed (5 mL PBS, 2X). The organisms remaining on each skin were recovered and quantitated as described using either the nitrocellulose lift alone or the nitrocellulose lift followed by skin homogenization (stomacher). Each experiment was performed in triplicate.

RESULTS & DISCUSSION

THE CONCENTRATION-DEPENDENT EFFECTS of various QAC on the viability of *S. typhimurium* grown in suspension were compared (Table 1). The EC_{50} (concentration producing 50% of maximum effect) for CPC, DTMAB, HTMAB, and TTMAB were calculated by means of a weighted nonlinear regression algorithm using data in Table 1 and are shown in Table 2. Based on the EC_{50} data the order of decreasing effect against *S. typhimurium* was CPC ≅ HTMAB > TTMAB >> DTMAB. A t-test analysis indicated that differences between the EC_{50}s were significant ($P \leq 0.001$).

The MIC's against *S. typhimurium* were determined for those QAC (CPC, DTMAB, HTMAB, TTMAB) with the greatest effects on viability of *S. typhimurium*. (see Table 2). The QAC with the lowest MIC was CPC which had an MIC of 40 µg/mL (1.1×10^{-4} M).

The viability data and MIC data cannot be compared directly because QAC activity on viability was calculated as an EC_{50} for each compound while the MIC data was the minimum concentration of QAC that caused 100% inhibition of visible growth. However, the relative order of activity of the QAC in both vi-

Table 1—Effect of quaternary ammonium compounds on viability of *Salmonella typhimurium*

Conc (μg/mL)	*Salmonella typhimurium* reduction (%)[a]			
	CPC	HTMAB	TTMAB	DTMAB
0	0.00	0.00	0.00	0.00
10	ND[b]	ND	3.60	9.40
20	89.11	71.00	18.50	ND
22.5	94.64	ND	ND	ND
25	99.70	ND	ND	ND
27.5	99.88	ND	ND	ND
30	99.95	ND	ND	ND
40	ND	99.68	95.33	ND
50	ND	ND	99.99	ND
60	ND	ND	99.99	ND
80	ND	99.99	99.99	ND
100	ND	99.99	ND	18.20
200	ND	ND	ND	25.10
250	ND	ND	ND	72.80
300	ND	ND	ND	99.97

[a] [(CFU/mL control − CFU/mL treated sample)/(CFU/mL control)] × (100); mean of three determinations.
[b] ND = not determined.

Table 2—Antimicrobial activities of quaternary ammonium compounds (QAC) on *Salmonella typhimurium* in suspension

QAC	EC50 ± SD[a] (× 10^{-6} M)	MIC Value[b] (× 10^{-4} M)
CPC	5.02 ± 0.19	1.11
HTMAB	4.88 ± 0.08	1.65
TTMAB	6.50 ± 0.10	1.78
DPC	ND[c]	2.65
DTMAB	75.00 ± 3.90	4.86

[a] Calculated from data in Table 1 using a nonlinear regression algorithm; EC50 is the molar concentration of QAC required to reduce *Salmonella* by 50%.
[b] The test (broth dilution method) was read by taking the least amount of QAC giving complete inhibition as judged by eye as the minimum inhibitory concentration (MIC).
[c] ND = Not determined.

ability and MIC studies was the same for the more active compounds (CPC, HTMAB, TTMAB) and CPC was the most active.

The bactericidal effects of QAC on *S. typhimurium* were measured to determine their relative order of bactericidal potency and to select compounds for subsequent studies of bacterial attachment to HBE cells and chicken skin. Viability determinations were performed with organisms suspended in buffered solutions, but MIC's were determined in broth. Models to evaluate the influence of QAC on attachment to HBE cells and chicken skin would involve both suspension and solution phenomena. Therefore, it was important to determine the influence of QAC on *S. typhimurium* in both media. Furthermore, determination of the concentration-dependent bactericidal effect of QAC against *S. typhimurium* provided the basis to distinguish the bactericidal effect from their effects on attachment.

The human buccal epithelial cell model described by Boussard et al. (1989) was utilized as a simple, rapid method for screening QAC. The model also was used to determine whether such effects were concentration dependent and whether there was structural selectivity.

A concentration-dependent action of the QAC was found in reversing attachment of *S. typhimurium* to HBE cells (Table 3). CPC was the only compound that produced a notable reversal of attachment at a concentration as low as 10 μg/mL. At that concentration it effectively removed an average of 65% of *S. typhimurium* from HBE cells. CPC was the most active QAC, followed in decreasing order by TTMAB, HTMAB, DPC, and DTMAB. The EC_{50}s for reversal of attachment of *S. typhimurium* to HBE were calculated (Table 4).

A concentration-dependent effect was found for CPC to inhibit attachment of *S. typhimurium* to HBE cells (Table 5). CPC at concentrations of 10 μg/mL and 100 μg/mL inhibited 68% of *S. typhimurium* attachment compared to untreated control cells. This was comparable to the results (Table 3) wherein 10 μg/mL CPC reversed attachment by 63%. Thus, in the HBE model, CPC was equally effective both in reversing and inhibiting attachment.

Table 3—Effect of quaternary ammonium compounds in reversing attachment of *Salmonella typhimurium* to human buccal epithelial cells (HBE)

Conc (μg/mL)	*Salmonella typhimurium* reduction (%)[a]				
	CPC	HTMAB	TTMAB	DTMAB	DPC
0	0	0	0	0	0
2.5	0	nd	nd	nd	nd
5	24	nd	nd	nd	nd
10	63	nd	nd	nd	nd
25	76	nd	27	nd	nd
50	nd[b]	21	57	nd	nd
75	nd	nd	nd	12	nd
100	nd	43	79	27	29
150	nd	nd	nd	31	33
175	nd	61	nd	nd	40
200	nd	nd	nd	41	nd
250	nd	77	nd	53	66
300	nd	nd	nd	50	74

[a] [(bacterial attached control − bacterial attached treated sample)/(bacterial attached control)] × (100); mean from 30–50 HBE cells.
[b] nd = Not determined.

Table 4—Activity of quaternary ammonium compounds (QAC) in reversing attachment of *S. typhimurium* to human buccal epithelial cells

QAC	EC_{50} ± SD[a] (× 10^{-5} M)	Correlation
CPC	2.5 ± 0.56	0.976
TTMAB	12.8 ± 0.64	0.998
HTMAB	33.2 ± 1.80	0.996
DTMAB	62.0 ± 11.5	0.944
DPC	63.0 ± 7.90	0.954

[a] Calculated from data in Table 3 using a nonlinear regression algorithm; EC50 is the molar concentration of QAC required to reverse attachment by 50%.

Table 5—Inhibition of attachment of *Salmonella typhimurium* to human buccal epithelial cells (HBE)

Cetylpyridinium chloride Conc (μg/mL)	Treated/Control[a]	% inhibition[b]
2.5	62/71	13
5	41/71	42
10	24/74	68
100	23/74	69

[a] Mean of attached bacteria per HBE cell; mean from 30–50 HBE cells.
[b] [(control − treated)/control] × (100).

The HBE cell model was selected because it represents a live-cell model of bacterial attachment, and it provides a laboratory model for facile quantitative evaluation of large numbers of chemical compounds with diverse structures and physicochemical properties. Of the cationic surfactant QACs tested, CPC proved most effective (lowest EC_{50}, Table 4) for reversing attachment of *S. typhimurium* to HBE cells. The EC_{50} for CPC was five times lower than the next most effective compound, TTMAB. HTMAB was the third most effective. CPC was extremely effective at inhibiting bacterial attachment to HBE cells, and the effect was also concentration dependent. Furthermore, at a concentration of 10 μg/mL, CPC was equally effective in reversing and inhibiting attachment.

The concentration of CPC required to effect ≈65% reversal and inhibition of *S. typhimurium* attachment to HBE was four times lower (10 μg/mL) than its MIC (40 μg/mL) against this organism. The EC_{50} (2.5×10^{-4} M) for reversing attachment of *S. typhimurium* to HBE cells was 1.7 orders of magnitude greater than its EC_{50} (5×10^{-6} M) against viability of this organism in suspension.

The effectiveness of two QAC (CPC and HTMAB), as well as trisodium phosphate (TSP) to both reduce and inhibit viable *S. typhimurium* attachment to chicken skin was measured using the novel Salari Chicken Skin Model (Breen et al., 1993) developed in our laboratory. This model used small pieces of chicken skin to reduce the number of birds required. It is safe, rapid, quantifiable, highly reproducible, and provides data that may be applied readily to statistical analyses. A concentration-

Table 6—Reduction of viable *Salmonella typhimurium* on chicken skin following treatment with cetylpyridinium chloride (CPC) at various exposure times and temperatures

CPC Conc (μg/mL)	*Salmonella typhimurium* recovered (CFU/mL)[a]					
	Nitrocellulose-Lift			Stomacher		
	60 min 25°C	60 min 35°C	60 min 45°C	60 min 25°C	60 min 35°C	30 min 35°C
0	80 ± 17	51 ± 8	85 ± 41	480 ± 240	4000 ± 1120	720 ± 320
5	ND[b]	ND	52 ± 13	ND	ND	ND
10	ND	17 ± 4**	ND	480 ± 160	4400 ± 400	640 ± 320
50	ND	22 ± 12**	71 ± 19	ND	ND	ND
100	37 ± 20*	18 ± 7**	ND	240 ± 240*	4000 ± 1040	480 ± 240
250	25 ± 15*	ND	ND	ND	ND	ND
500	29 ± 21*	ND	24 ± 13	ND	ND	ND
1000	15 ± 18**	7 ± 2**	28 ± 19	0**	640 ± 560**	400 ± 240

[a] *Salmonella* recovered either by nitrocellulose lift technique or stomacher digestion of each skin; data are the mean (± SD) of three determinations. Statistical analysis performed using ANOVA followed by Newman-Keuls.
[b] ND = Not determined.
* $P < 0.05$ Compared to control, ** $P < 0.01$ Compared to control.

Table 7—Reduction of viable *Salmonella typhimurium* on chicken skin following treatment with hexadecyltrimethylammonium bromide (HTMAB) or trisodium phosphate (TSP) at various exposure times at room temperature

Treatment Conc (mg/mL)	*Salmonella typhimurium* recovered (CFU/skin)[a]							
	Nitrocellulose-Lift				Stomacher			
	Exposure time, min				Exposure time, min			
	0.25	3.0	10.0	30.0	0.25	3.0	10.0	30.0
Control	52 ± 18	81 ± 32	130 ± 35	146 ± 41	240 ± 160	400 ± 240	320 ± 160	640 ± 240
HTMAB 0.01	ND[b]	ND	149 ± 29	128 ± 51	ND	ND	400 ± 160	640 ± 480
HTMAB 0.1	ND	ND	122 ± 8	123 ± 14	ND	ND	320 ± 160	320 ± 240*
HTMAB 1	ND	ND	70 ± 37	64 ± 12*	ND	ND	80 ± 80**	26 ± 80**
TSP 1	79 ± 15	79 ± 16	ND	ND	400 ± 160	400 ± 240	ND	ND
TSP 10	60 ± 7	39 ± 4*	ND	ND	320 ± 160	400 ± 240	ND	ND
TSP 100	13 ± 2*	0*	ND	ND	80 ± 80*	0**	ND	ND

[a] *Salmonella* recovered using nitrocellulose lift technique followed by digestion of each skin with a stomacher; data are the mean (±SD) of three determinations. Statistical analysis performed using ANOVA followed by Newman-Keuls.
[b] ND = Not determined.
* $P < 0.05$ Compared to control, ** $P < 0.01$ Compared to control.

dependent effect of CPC, and the effect of different incubation temperatures and times were compared in reducing viable *S. typhimurium* on chicken skin (Table 6) using this model. When inoculated skin was exposed to CPC at 35°C for 60 min, 10 μg/mL of CPC reduced viable *S. typhimurium* to 33% of control ($p < 0.01$) using the nitrocellulose lift technique to recover attached *S. typhimurium*. Attachment of viable organisms was reduced to 14% of control ($p < 0.01$) at 1000 μg/mL. *S. typhimurium* was reduced to 46–57% (depending upon inoculum) of control ($p < 0.05$) at 100 μg/mL CPC (25°C) and to 14–19% of control ($p < 0.01$) at 1000 μg/mL (25°C). No reduction was observed when exposure temperature was 45°C.

When *S. typhimurium* was recovered using the Stomacher (Table 6), treatment of skin with 100 μg/mL CPC at 25°C for 60 min reduced *S. typhimurium* 50% ($p < 0.05$ compared to control). This was similar to the effects when *S. typhimurium* was recovered by nitrocellulose lift. Treatment with 1000 μg/mL CPC under the same conditions provided 100% reduction ($p < 0.05$ compared to control) of *S. typhimurium*. Incubation of skin at 35°C, for 60 min with 1000 μg/mL CPC reduced *S. typhimurium* to 16% of control ($p < 0.05$). This indicated that the higher incubation temperature reduced slightly the ability of CPC to reduce viable *Salmonella typhimurium*.

The effects of HTMAB and trisodium phosphate (TSP) on reducing viable *S. typhimurium* on chicken skin were compared (Table 7). HTMAB treatment at 35°C for 30 min had an effect comparable to that of CPC under identical conditions. The effect of TSP approximated that of CPC only when the skin was treated with concentrations 100 times that of CPC (100 mg/mL TSP versus 1 mg/mL CPC) for 3 min at 25°C. Under those conditions, deleterious effects on skin texture and color were observed. The texture was slick and rubbery, and the skin lost its color. Such adverse skin changes were more apparent when the skin was treated with this concentration of TSP for longer times. Treatment with TSP for a shorter time (15 sec) produced no deleterious effects on skin texture and color, but were somewhat less effective in reducing attachment. Lower TSP concentrations (<100 mg/mL) for longer exposure times (≥ 30 min.) were not evaluated. TSP appears to exert action *via* high pH conditions, which would exert physical changes on the protein of the skin.

The CPC was effective on inhibition of *S. typhimurium* attachment to chicken skin (Table 8). Note that in all inhibition experiments skins were thoroughly washed to remove the test compound prior to inoculation with bacteria. Pretreatment for 10, 30, and 60 min at room temperature with 100 μg/mL of CPC inhibited *S. typhimurium* attachment, giving 24, 17, and 22%, respectively, of control levels ($p < 0.05$) when bacteria were recovered by nitrocellulose lift. When skins were pretreated with 1000 μg/mL CPC under the same conditions, inhibition of attachment was virtually complete. These data indicate further that pretreatment time had no influence on the ability of CPC to inhibit attachment at the two concentrations. Pretreatment for 30 min at room temperature with 10 μg/mL CPC inhibited *S. typhimurium* attachment by 50% when bacterial recovery was by Stomacher digestion ($p < 0.05$). A dose-response correlation was seen, with pretreatment at 1000 μg/mL CPC causing virtually complete inhibition of *S. typhimurium* attachment regardless of method used to recover bacteria.

The effects of HTMAB on inhibition of *S. typhimurium* attachment to chicken skin were also compared (Table 9). Regardless of recovery method, at both 100 μg/mL and 1000 μg/mL it was comparable to CPC when incubated for the same time at the same temperature.

The effect of TSP on inhibition of *S. typhimurium* attachment to chicken skin was compared (Table 10). Pretreatment of skin for 10 min with TSP gave 100% inhibition of attachment, but only at a concentration 100 times greater (100 mg/mL) than the equally effective concentration (1 mg/mL) of CPC. This inhibitory effect of TSP was completely abolished when the skins were washed following treatment, as was done with CPC and HTMAB, before inoculation with bacteria. A comparison of

Table 8—Inhibition of *Salmonella typhimurium* attachment to chicken skin pre-treated with cetylpyridinium chloride (CPC) at various exposure times at room temperature

Recovery Method	CPC expos. Time (min)	*Salmonella typhimurium* recovered (CFU/skin) CPC concentration (μg/mL) 0	10	100	1000
Nitro.-Lift	10	106 ± 45	113 ± 31	25 ± 35*	1 ± 1**
	30	144 ± 31	144 ± 24	25 ± 21**	1 ± 1**
	60	169 ± 56	156 ± 55	38 ± 16**	0**
Stomacher	30	960 ± 480	480 ± 160*	80 ± 80*	0**
	60	1120 ± 320	720 ± 720	80 ± 240*	0*

[a] *S. typhimurium* recovered using nitrocellulose lift technique followed by digestion of each skin with a stomacher; data are the mean (+SD) of three determinations. Statistical analysis performed using ANOVA followed by Newman Keuls.
* $P < 0.05$ Compared to control, ** $P < 0.01$ Compared to control.

Table 9—Inhibition of *Salmonella typhimurium* attachment to chicken skin pre-treated with hexadecyltrimethylammonium bromide (HTMAB) at different exposure times

Recovery method	HTMAB expos. Time (min)	*Salmonella typhimurium* recovered (CFU/skin)[a] HTMAB Concentration (μg/mL) 0	10	50	100	1000
Nitro.-Lift	30	120 ± 35	83 ± 17	ND[b]	21 ± 12**	0**
	60	130 ± 9	71 ± 27*	26 ± 22**	6 ± 1**	0**
Stomacher	30	320 ± 160	320 ± 240	ND	80 ± 80**	0**

[a] *S. typhimurium* recovered using nitrocellulose lift technique followed by digestion of each skin with a stomacher; data are the mean (±SD) of three determinations. Statistical analysis performed using ANOVA followed by Newman-Keuls.
[b] ND = Not determined.
* $P < 0.05$ Compared to control, ** $P < 0.01$ Compared to control.

Table 10 Inhibition of *Salmonella typhimurium* attachment to chicken skin pre-treated with trisodium phosphate (TSP) at different exposure times

Recovery method	TSP expos. Time (min)	*Salmonella typhimurium* recovered (CFU/skin)[a] TSP Concentration (mg/mL) 0	1	10	100
Nitro.-Lift	0.25	151 ± 74	124 ± 45	160 ± 31	162 ± 87
	10[b]	152 ± 32	138 ± 17	26 ± 19**	0**
Stomacher	0.25	480 ± 160	480 ± 240	480 ± 160	320 ± 160
	10[b]	960 ± 400	880 ± 240	80 ± 160**	0**

[a] *S. typhimurium* recovered using nitrocellulose lift technique followed by digestion of each skin with a stomacher; data are the mean (±SD) of three determinations. Statistical analysis performed using ANOVA followed by Newman-Keuls.
[b] Skins not washed following pre-treatment with TSP.
* $P < 0.05$ Compared to control, ** $P < 0.01$ Compared to control.

TSP (Table 10) and CPC (Table 8) for the same exposure time (10 minutes) revealed that the concentration of TSP that was effective (100 mg/mL) caused deleterious physical changes. In contrast, 100 μg/mL CPC prevented 76% contamination ($p < 0.05$) and 1000 μg/mL CPC prevented 99.1% contamination ($p < 0.01$). The ability of TSP to inhibit attachment, as well as its reduction of viable organisms, may be due to the extremely high pH (pH 12).

By comparison of experimentally determined EC_{50}'s, the greatest effectiveness of CPC was on viability of *S. typhimurium* in broth. Virtual elimination of 10^9 CFU/mL *S. typhimurium* in broth was accomplished by the addition of 0.005% CPC for 30 sec.

In the buccal epithelial cell study, microscopy of stained cells showed physical detachment of *Salmonella* by CPC and other quaternary ammonium compounds. The three criteria of exposure time, initial bacterial load, and experimentally determined EC_{50}s indicated that the process of removal of *Salmonella* from human buccal epithelial cells was more difficult than killing *Salmonella* in broth, but less difficult than reversal of *Salmonella* contamination of chicken skin. Moreover, it appears that physical removal of *Salmonella typhimurium* from tissue by CPC, either subsequent to or in addition to the antibacterial effect is important in the reversal of *Salmonella* contamination of tissue.

Cationic surfactant QAC's are known to have bactericidal action on inanimate surfaces, in suspension, and on human tissues. However, a more stringent and pertinent test of their food safety efficacy was the chicken skin model we employed. The two techniques employed to recover bacteria, nitrocellulose lift and Stomacher digestion, might be expected to collect the largest possible amounts of bacteria, which would help insure reproducible results. The Stomacher technique may be viewed as the criterion for retrieval of bacteria, even those most deeply imbedded in chicken skin. That technique would allow collection of bacteria not removed by nitrocellulose lift, which itself is a retrieval method more effective than either swabbing or rinsing techniques.

In both reduction and inhibition experiments on excised chicken skin, where bacteria were retrieved by nitrocellulose lift and Stomacher techniques, CPC and HTMAB had comparable effectiveness. CPC and HTMAB were shown by this model, in a highly quantifiable way, to be effective at reducing viable *S. typhimurium* on chicken skin in a concentration-dependent manner. CPC and HTMAB were more effective at inhibiting contamination of chicken skin, when skin was exposed to these QAC's for short exposure times and the skins had been washed thoroughly before being inoculated with bacteria. TSP, although effective in high concentration at inhibiting and reducing *S. typhimurium*, had limitations to its use that cationic surfactants did not have. Extremely high pH, lack of efficacy when washed off, and undesirable changes in skin color, texture, and appearance were observed when it was utilized.

CONCLUSIONS

IN LABORATORY SCALE MODELS for measuring attachment of viable *S. typhimurium* to chicken skin QAC, especially CPC and HTMAB, were extremely effective both in reducing and inhibiting attachment. Their inhibition and reduction of attachment of *S. typhimurium* appeared to be structurally related. Concentrations at which they produced these effects were extremely low, much lower than the concentration for TSP to produce the

same effects. Our results suggest that QAC, and especially CPC, might be useful agents to prevent or reduce *S. typhimurium* contamination of poultry carcasses. These compounds would likely also prevent or reduce bacterial cross-contamination of carcasses.

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Ms received 12/23/94; revised 6/7/95; accepted 6/23/95.

We thank Robert W. Moore, Univ. of Arkansas Poultry Processing Plant, Fayetteville, for providing chicken skins. We acknowledge the most helpful suggestions from Dr. P. Broussard Institut de Pharmacie of the Universite Libre de Bruxelles in Brussels, Belgium, regarding our application of the human buccal epithel al cell model.

This research was supported in part by a grant from the USDA CSRS to the Food Safety Consortium.

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Ms received 3/16/95; revised 6/1/95; accepted 6/23/95.

Contribution No. 95-341-J from the Kansas Agr. Exp. Station, Manhattan, KS 66506.

This research was supported in part by the Kansas Value Added Center. We thank Dr. Ram Prasi for performing the microbiological analyses and Dr. Dan Schaefer, Kristine Scheller, and Qingping Liu for assistance in procuring meat and for performing the α-tocopherol analyses.

Volatile Content and Sensory Attributes of Supercritical Carbon Dioxide Extracts of Cooked Chicken Fat

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ABSTRACT

Volatile and sensory profiles were generated for extracts of cooked chicken fat obtained with supercritical carbon dioxide at 10.3 MPa, 20.7 MPa, and 31.0 MPa at 40°C. Volatiles (318 total) were quantitated and 77 were identified. Concentrations of total volatiles, 7 compound classes, and 63 individual compounds were affected by treatments, generally increasing with decreasing extraction pressure. All extracts had higher concentrations than unextracted control. Total volatiles were concentrated 12-fold. Six sensory notes differed by treatment. Chicken fat aroma and flavor intensities were greater for all extracts than control and increased as pressure decreased. Total volatiles, 5 classes, and 42 individual compounds correlated with chicken fat aroma and/or flavor.

Key Words: chicken fat, sensory, volatiles, supercritical CO_2

INTRODUCTION

THE DEMAND FOR LOW-FAT PRODUCTS which often have inherent flavor disadvantages, especially in meats area require development of flavor concentrates for potential use in such products. Supercritical fluid extraction has potential for producing natural flavor concentrates while using excess lipid by-products of the poultry industry.

Supercritical fluids are heated and pressurized beyond the fluid-specific critical temperature and pressure (Bott, 1982; Calame and Steiner, 1982). They have solvating ability like liquids and diffusivity and mass transfers similar to gases (Brogle, 1982; Caragay, 1981; de Filippi, 1982; Hardardottir and Kinsella, 1988). Carbon dioxide is a popular supercritical fluid because it is low cost, nonflammable, nontoxic, and has relatively low critical temperature (31.1°C) and pressure (7.38 MPa). Commercial utilization of this technology includes decaffeination of coffee beans, and obtaining hops extracts and spice and fruit juice essential oils. Total extractions, removal of specific components, fractionation of fats and oils, and concentration of aroma and flavor compounds have been reported (Rizvi et al., 1986).

de Haan and de Graauw (1990) concentrated flavor compounds from milk fat up to 50-fold. Vapor pressure differences between volatiles and nonvolatile triglycerides facilitated extraction. Concentration of raw beef fat volatiles was demonstrated by Merkle and Larick (1994) with noted increases in volatile concentrations as extraction pressure decreased. Higher molecular weight volatiles became more soluble in the supercritical fluid as extraction pressure increased (Shimoda et al., 1994).

Over 300 volatile compounds have been identified from chicken systems, but chicken flavor is associated primarily with hydrocarbons, aldehydes, enals, ketones, and sulfur compounds. Aldehydes, enals, and sulfur-containing compounds have been most important. Minor et al. (1965) associated carbonyls with "chickeny" flavor and sulfur compounds with "meaty" flavor. Specifically, 2-alkenals including hexenal, heptenal, octenal, nonenal, undecenal, and dodecenal as well as aldehydes like octanal, nonanal, and decanal along with decadienal and γ-dodecalactone have been indicated to have potential impact on chicken-specific aroma and flavor (Gasser and Grosch, 1990; Pippen and Nonaka, 1960; Ramarathnam et al., 1991).

Species-specific notes are generally lipid-derived while meaty notes are lean-derived (Dwivedi, 1975; Hornstein and Crowe, 1960; 1963; Minor et al., 1965; Shahidi et al., 1986). Differences between species' fatty acid profiles and resulting carbonyls may be responsible for the lipid influence (Allen and Foegeding, 1981; Gray et al., 1981; Kim Ha and Lindsay, 1990; Ramarathnam et al., 1991; Ramaswamy and Richards, 1982; Rubin and Shahidi, 1988; Shahidi et al., 1986; Wong et al., 1975a,b). Dietary lipids are a primary determinant of poultry fatty acid deposition (Jen et al., 1971; Schuler and Essary, 1971). Lipids may dissolve or adsorb and later release important aroma/flavor compounds or be degraded via thermal and oxidative reactions to have sensory effects (Dwivedi, 1975; Moody, 1983; Selke et al., 1975; Shahidi et al., 1986).

Our objectives were to study supercritical fluid extraction to concentrate volatile compounds of chicken fat and to characterize important compounds for chicken sensory notes.

MATERIALS & METHODS

Sample preparation

Raw chicken fat was collected from random processing lots at the halving machine from a commercial processor, manually freed of nonlipid material and ground once through a 0.95 cm plate. Portions (500g) were weighed and vacuum-packaged in low permeability bags (20 × 28 cm P641B clear pouches; Cryovac Corp., Duncan, SC) and overwrapped with polyethylene coated freezer paper. Samples were stored at −20°C and thawed at 2–4°C prior to use. Prior to extraction, fat was heated in a convection oven (30 min) to 80°C in 500 mL Pyrex glass beakers to melt the triglyceride portion and create a substrate mimetic of roasted chicken fat. An unextracted portion was placed in a Pyrex tube (16 mm × 125 mm), flushed with N_2 gas, sealed with a teflon-lined cap, frozen at −10°C and retained as a control.

Extraction

A Superpressure model 46-13421-2 supercritical fluid extractor (Newport Scientific, Jessup, MD) equipped with a 69.0 MPa double end diaphragm compressor was used to fractionate the cooked chicken fat. Aliquots (250g) were immobilized between 2 plugs of glass wool and loaded into a 0.845L (internal volume) stainless steel extraction vessel. Vessel temperature was maintained at 40°C via an internal thermocouple and temperature controller with heat tape wrapped around the outside of the vessel. Stainless steel transfer lines were wrapped with insulation to maintain temperature within the system. Continuous extractions were carried out at 10.3, 20.7, and 31.0 MPa, using SC-CO_2 at 10–15 L/min to a total flow volume of 500 L ambient CO_2 measured using a flow totalizer. Extracts were collected in a 500 mL Pyrex round bottom flask upon fluid depressurization at ambient temperature. Extracts were transferred to Pyrex tubes (16 mm × 125 mm), flushed with N_2, sealed with a teflon-lined cap, and frozen at −10°C until analysis.

Volatile quantitation

Extracts and unextracted control were melted in a 60°C water bath. Samples (300 mg) were placed into another tube and 1026 ng internal standard, 2,3,4-trimethylpentane, was added. The tube was sealed and vortexed. Aliquots (100 mg) were placed between 2 plugs of pesticide grade glass wool in a 9 mm × 85 mm glass tube. With a 6-port sample

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Table 1—Volatile class concentrations of supercritical carbon dioxide extracts of chicken fat

	Pressure (MPa)	Concentration (ppm) 0.0	10.3	20.7	31.0
Class					
Enals		1.37^{c}	15.75^{a}	10.65^{ab}	4.06^{bc}
Aldehydes		1.43	16.32	11.96	6.09
Ketones*		0.11^{b}	3.21^{a}	2.82^{a}	1.06^{b}
Lactones*		0.16^{d}	4.43^{a}	1.77^{b}	0.85^{c}
Alkanes		0.14	1.70	1.06	0.69
Branched alkanes		4.26^{b}	26.79^{a}	14.45^{b}	9.10^{b}
Alcohols		0.05^{b}	3.60^{a}	2.04^{ab}	0.80^{b}
Acids		0.52	3.47	2.18	0.04
Alkenes		0.53	4.40	3.16	2.27
Halogenates		0.06^{b}	0.70^{a}	0.54^{a}	0.40^{ab}
Aromatics		0.06^{c}	1.65^{a}	1.15^{ab}	0.57^{bc}
Heterocyclics		0.00	0.04	0.00	0.07
Total*		9.94^{c}	123.80^{a}	76.63^{ab}	37.49^{bc}

^{abcd} Means in same row with same letter or with no superscript do not differ significantly ($P \leq 0.05$).
* ($P \leq 0.01$).

valve in the no-flow position, the tube was positioned in an External Closed Inlet Device (Scientific Instrument Service, River Ridge, LA). Volatiles were stripped from the sample via this method of direct thermal desorption followed by cold trapping for 5 min. Temperatures were: inlet 150°C, valve 160°C, and carrier lines 170°C. Volatiles were flushed onto a nonpolar 30m DB-5 fused silica capillary column (J & W Scientific, Folsom, CA) with internal diameter 0.32 mm and film thickness 1.0 micron. The column was in a Varian 3700 gas chromatograph (Varian, Palo Alto, CA) equipped with a flame ionization detector and maintained with head pressure of 1.13 psi, helium carrier gas flow rate of 5.73 mL/min, and split ratio of 20.12:1. An oven temperature program of −30°C to 290°C at 4°C/min was used with a 1 min hold at −30°C. Data were analyzed using the Maxima 820 Chromatography Workstation (Millipore, Waters Chromatography Division, Milford, MA). Quantitation of volatiles was based on relative peak areas compared to peak area of internal standard.

Volatile identification

For identification via electrical ionization, 10.3 MPa and 20.7 MPa extract samples (200 mg or 550 mg plus 1 mL deionized water) were placed into a sampling tube of a Tekmar LSC-3 headspace concentrator. Samples were purged at 110°C for 14 to 20 min followed by 5 min desorption onto a 30m DB-5 fused silica capillary column with internal diameter 0.32 mm and film thickness 1.0 micron within a Hewlett Packard 5985 gas chromatograph/mass spectrometer. Oven temperature program was identical to that used in volatile quantitation and ionization potential was 70 eV. Scan range was 40–300 atomic mass units. For chemical ionization with methane gas, samples (500 mg) were purged 14 min, desorbed 5 min, and scanned over a 40–300 atomic mass unit range with the same equipment set-up and an ionization potential of 230 eV.

For more efficient concentration of higher molecular weight compounds, a modified liquid/liquid extraction was used (Likens and Nickerson, 1964). Cooked fat (100g) and deionized water (150 mL) were placed into a 500 mL Pyrex round bottom flask with boiling chips and hooked to the long arm of a water-jacketed codistillation apparatus. The short arm was fitted to a 100 mL Pyrex round bottom flask containing boiling chips and 75 mL Optima grade methylene chloride (Fisher Scientific, Fair Lawn, NJ). Heating mantles beneath each flask were used to heat samples to boiling. Once both sides had begun condensing at the top of the still, codistillation was allowed for 6 hr. After codistillation and cooling, remaining solvent phase containing entrapped volatiles was drained from the distillation apparatus and added to the 100 mL Pyrex round bottom flask. A Buchi rotary evaporator was used to concentrate solvent fraction to ≈ 1 mL. Flask was washed 3 times with 1 mL methylene chloride and the washings were added to original 1 mL of concentrate. Combined sample was concentrated under a gentle stream of N_2 to 0.1 mL prior to GC-MS analysis.

Samples (1-2 µL) from liquid/liquid extractions were injected directly onto a 30m DB-5 capillary column in a Hewlett Packard 5985 gas chromatograph/mass spectrometer under conditions noted and scanned over a 40–500 atomic mass unit range. Similar conditions were used for chemical ionization with methane gas with an ionization potential of 230 eV as for previous chemical ionization analyses. Volatile identifications were based on instrument retention times for previously identified compounds and GC-MS with both electrical and chemical ionization methods and comparison to NIH/EPA (1978) reference spectra.

Sensory analysis

Separate extractions for each treatment under identical conditions, using food-grade carbon dioxide, were carried out to collect samples for sensory analysis. Extracts were flushed with nitrogen gas and stored at −10°C.

Melted extracts and control were presented as a 30% solution in mineral oil to an established 7 member trained flavor profile panel (ASTM, 1981; Caul, 1957). Prior chicken training sessions (3) included samples of melted (as described above) chicken fat, 10.3 MPa extract, pure mineral oil, and cooked chicken meat. Treatment samples were evaluated individually using a 14 point intensity scale (1 = not detectable, 14 = strong) on 5 aroma and 9 flavor notes previously selected by panelists during training or dictated by experimental goals. Consensus scores were developed through discussion by all panelists following independent evaluation. Panel evaluation was completed in 2 sessions with 4 samples/session.

Experimental design and statistical analysis

Extractions and volatile profiles were replicated 3 times on different samples of fat for the 4 treatments, 3 extracts and unextracted control. Extractions and sensory profiles were replicated twice on fat from a single source for the 4 treatments. Volatile concentrations and sensory responses were analyzed via analysis of variance using a randomized complete block design with replicates as blocks. Waller-Duncan k-ratio t-tests were used to separate means (SAS Institute, Inc., 1990). Volatile concentrations and sensory responses were averaged across replicates and correlated using Pearson correlation coefficients (SAS Institute, Inc., 1990). All reported significant differences and correlations are at $P \leq 0.05$, unless otherwise noted.

RESULTS & DISCUSSION

Volatile quantitation and identification

Quantitated volatile compounds numbered 318 of which 99 were grouped into 12 compound classes—enals, aldehydes, ketones, lactones, alkanes, branched alkanes, alcohols, acids, alkenes, halogenated compounds, aromatic hydrocarbons, and heterocyclics. Of the 99 compounds, 77 were identified. All enals, aldehydes, alkanes, acids, and aromatic hydrocarbons and some ketones, lactones, alcohols, alkenes, halogenated compounds, and similar heterocyclics have been noted in other chicken samples (Gasser and Grosch, 1990; Katz et al., 1966; Shahidi et al., 1986). Thermal breakdown of fatty acids and oxidation of unsaturated lipids could yield compounds representative of all volatile classes (Ellis et al., 1961; Hoffman, 1962; MacLeod and Seyyedain-Ardebili, 1981) except aromatic hydrocarbons and halogenated alkanes. They likely came from the breakdown of aromatic amino acids in the original adipose tissue or feed constituents and entrapped water, respectively. The largest numbers of classified volatiles were branched alkanes (38), enals (12), and aldehydes (9) followed by alkenes (8), alcohols (6), ketones (6), aromatic hydrocarbons (5), halogenated compounds (4), lactones (4), alkanes (3), acids (3), and heterocyclics (1). An additional 11 volatile compounds (2-propenal; pentane; carbon disulfide; 2-methylpentane; nitromethane; 3-methylpentane; 2-methyl-1-pentene; butanal; hexane; 1,2-dichloroethene; and chloroform) were identified in extracts. However they were not quantitatable due to the coelution of internal standard carrier solvent, hexane.

By concentration, branched alkanes, aldehydes, and enals were predominant (Table 1) in all treatments. Treatments affected total volatile concentration which was higher for all extracts vs control and, among extracts, increased with decreasing extraction pressure. Total volatiles were concentrated over 12-fold in 10.3 MPa extract vs control. Similarly, concentrations of enals, ketones, lactones, branched alkanes, alcohols, and halogenated and aromatic compounds were affected by treatment. Concentration of aldehydes followed a similar trend but varia-

Table 2—Pearson correlation coefficients for identified volatiles and flavor notes that differed by treatment

Peak No.	Identification	Chicken fat flavor	Astringent flavor
50	4-Methyloctane	0.972;0.028[a]	**[b]
66	2,2,4-Trimethylheptane	**	0.979;0.020
69	2,2,6-Trimethylheptane	**	0.982;0.018
70	2-Heptanone	0.975;0.025	**
88	1-Nonen-3-ol	**	**
89	2,3-Octanedione	**	**
91	2-Pentylfuran	**	0.998;0.002
108	(2,5 or 2,6)-Dimethylundecane	**	**
130	Nonanal	**	0.999;0.001
137	(2,2,6 or 2,2,7)-Trimethyldecane	**	**
147	2(E)-Nonenal	**	0.999;0.001
155	3,3-Dimethylundecane	**	0.972;0.028
164	Decanal	**	0.984;0.016
165	2,4-Nonadienal	**	0.992;0.008
201	2-Undecenal	**	0.967;0.033
211	2,5-Dimethyldodecane	**	0.998;0.002
244	2,6-Di-*t*-butyl-4-methylphenol	**	**
264	Tetradecanal	**	0.959;0.042
273	γ-Dodecalactone	**	0.951;0.049
274	2-Pentadecanone	**	0.987;0.013
275	5-Propyltridecane	**	0.977;0.023
277	δ-Dodecalactone	**	**
290	Hexadecanal	**	0.970;0.031
300	2-Heptadecanone	**	**
306	δ-Tetradecalactone	**	**

[a] Correlation coefficient; level of significance.
[b] Not significant (P≤0.05).

Table 3—Aroma and flavor responses of supercritical carbon dioxide extracts of chicken fat

	Sensory response[d]			
	Pressure (MPa)			
Note	0.0	10.3	20.7	31.0
Oxidized aroma	1.1[b]	3.5[a]	3.4[a]	3.3[a]
Chicken fat aroma	1.8[b]	3.8[a]	3.1[a]	2.8[ab]
Mineral oil aroma	1.6	1.5	1.7	1.4
Cooked aroma	1.1	1.2	1.1	1.1
Meaty aroma	1.0	1.0	1.0	1.0
Oxidized flavor*	1.0[b]	4.3[a]	4.1[a]	4.5[a]
Chicken fat flavor	2.3[c]	4.6[a]	4.1[ab]	3.8[b]
Mineral oil flavor	1.9	1.7	1.6	1.8
Metallic flavor	1.0	1.6	1.3	1.3
Cooked flavor	1.3	1.8	1.5	1.1
Sour flavor	1.6	1.8	1.7	1.8
Astringent flavor	1.8[b]	2.4[a]	2.1[ab]	1.9[b]
Meaty flavor	1.1	1.1	1.0	1.0
Oxidized aftertaste*	1.0[b]	3.8[a]	3.6[a]	4.0[a]

[abc] Means in same row with same letter or with no superscript do not differ significantly (P≤0.05).
[d] Scored on a 1–14 scale.
* (P≤0.01).

Table 4—Identified volatiles correlated with chicken fat aroma and flavor

Chicken fat aroma	Chicken fat flavor
Pentanal	1,1,1-Trichloroethane
Hexanal	2,2,4-Trimethylhexane
2-Octene	2-Octene
4-Methyloctane	2-Methyl-2-heptene
3,5-Dimethylheptane	4-Methyloctane
(1,3 or 1,4)-Xylene	2,6-Dimethylheptane
2,2,5-Trimethylheptane	3,5-Dimethylheptane
2,2,4-Trimethylheptane	(1,3 or 1,4)-Xylene
2,2,6-Trimethylheptane	2-Heptanone
2-Heptanone	
Xylene isomer	
Heptanal	
2(E)-Decenal	
Tetradecane	
2-Methyl-6-propyldodecane	

tion between replicates, partially due to variation in fat source, limited significance (P≤0.10). Classes were concentrated from 6-fold for branched alkanes to 66-fold for alcohols with enals and aldehydes concentrated 11-fold. The concentrations achieved were related to both the nature of the class of compounds with regard to volatility and polarity as well as specific compounds identified. The concentration of aldehydes, enals, and lactones was expected as they should be quite soluble in SC-CO_2. The branched alkanes are more volatile and soluble than their straight chain counterparts. More polar alcohols should be less soluble but several of those identified had unsaturation which would increase solubility. Halogenated compounds noted consisted of very short carbon chains. The aromatic compounds were all monocyclic with the only phenol having considerable branching to enhance solubility (Dandge, 1985). Merkle and Larick (1994) documented similar results with supercritical fluid extracted raw beef fat.

Individual compounds affected by treatments numbered 63 of which 25 were identified—7 branched hydrocarbons, 4 aldehydes, 4 ketones, 3 enals, 3 lactones, 2 aromatics, 1 alkene, and 1 alcohol (Table 2). All compounds except 2 (2,3-octanedione and 2,6-di-*t*-butyl-4-methylphenol) were in greater concentration in all extracts vs control and concentration increased with decreasing extraction pressure. These 2 compounds were more concentrated in all extracts than in the control, with the greatest concentration in the 20.7 MPa extract. 2,6-di-*t*-Butyl-4-methylphenol was identified in fatty acid analyses and its concentration increased (P≤0.10) with decreasing extraction pressure (Taylor, 1994). Ramarathnam et al. (1993) identified this compound as unique to chicken compared to beef and pork.

Nonanal, 2(E)-nonenal, 2(E),4(E)-nonadienal, 2-undecenal, and γ-dodecalactone have been identified as potent odorants with mainly "fatty" and "tallowy" descriptions (Gasser and Grosch, 1988, 1990; Ullrich and Grosch, 1987). Nonanal, 2-undecenal, and γ-dodecalactone were indicated as having specific odor potency in chicken over bovine species. Decanal, also, had notably higher concentration in chicken than in beef and pork (Ramarathnam et al., 1991).

The largest concentrating effect of extraction was for 2-heptadecanone and trimethyldecane (2,2,6- or 2,2,7-). Individual enals and aldehydes, noted as important to chicken attributes, were concentrated 17-fold for nonanal to 68-fold for 2,4-nonadienal and γ-dodecalactone increased 29-fold.

Hexanal was in highest concentration in all treatments. It increased (P≤0.10) with decreasing extraction pressure though all extracts had more than did the control. 2(E),4(Z)-Decadienal was next highest in all treatments except the 10.3 MPa extract which had 2-undecenal at the second highest concentration. Hexanal is a likely product of oxidation of linoleic acid or further oxidation of preformed 2,4-decadienal via an epoxide intermediate. 2,4-Decadienal could arise from carbon-carbon bond cleavage on the chain of the 9-hydroperoxide arising from linolenic acid (18:3). Cleavage of the carbon-carbon bond on the acid side of the 8-hydroperoxide radical from the oxidation of oleic acid (18:1) could account for the presence of 2-undecenal (Fennema, 1985).

All pressure treatments had sufficient solvating capacity in the SC-CO_2 to result in volatile concentration over the unextracted control. The volatile concentration trend was due to the supercritical fluid's decreased solubilization at lower pressures for less volatile and higher molecular weight fatty acids and triglycerides. Thus, volatiles were selectively extracted and concentrated. At higher pressures, more types of compounds would compete for position in the supercritical fluid phase. Merkle and Larick (1994) similarly achieved concentration of beef fat volatiles at lower pressures. Near the critical point of CO_2, on the steeper portion of the solubility curve, is where the most effective separations should occur (Allada, 1984).

Sensory analysis

Five aroma and 9 flavor notes were evaluated by trained panelists (Table 3). All notes, except meaty descriptors, were placed on the ballot by panelists during training. The meaty note was

added to ascertain if a purely lipid substrate would produce meaty sensations.

Among aroma notes, oxidized and chicken fat notes differed with treatment. All extracts elicited greater response intensity of these notes from panelists than did unextracted control. A trend of increasing intensity with decreasing pressure among extracts was noted, especially for the chicken fat aroma note. No meaty aroma and very little cooked aroma were detectable in any treatment, indicating the inability of a cooked lipid substrate to produce volatiles required for meaty notes, concurring with reported studies (Dwivedi, 1975; Hornstein and Crowe, 1960, 1963; Minor et al., 1965; Shahidi et al., 1986).

Among flavor notes, oxidized, chicken fat, astringent, and oxidized aftertaste were greater in all extracts than in controls with the exception of the 20.7 MPa and 31.0 MPa treatments for the astringent note. Chicken fat and astringent flavor increased among extracts with decreasing extraction pressure with a very clear trend for the chicken fat note. The meaty note was barely detectable in any sample, on average, and not by all panelists in any case. Clearly, intensity of aroma and flavor responses, especially for oxidized and chicken fat notes, increased with extractions. Their values increased several levels by extraction and usually in sequence, indicating the close association of such perceptions. Oxidized aroma and flavor, often undesirable attributes, increased but we could not determine from the data if the increase reached unacceptable levels as no hedonic testing was undertaken. The 30 % solution presented to panelists was well in excess of the usage level common for flavor concentrates but was required for adequate numerical data to facilitate distinction of differences.

Correlation of volatile concentrations and sensory notes

Qualitatively, trends for chicken fat aroma and flavor and oxidized aroma were very similar to concentration trends for total volatiles, branched alkanes, aldehydes, and enals. Total volatiles, aldehydes, alkanes, alkenes, halogenated compounds, and aromatic hydrocarbons correlated with chicken fat aroma. A negative correlation for heterocyclics and mineral oil aroma was noted. Alkenes and halogenated compounds correlated with chicken fat flavor. Lactones, alkanes, and branched alkanes correlated with metallic flavor. Acids correlated with cooked flavor. All classes except ketones and halogenated and heterocyclic compounds correlated with astringent flavor.

Correlations for concentrations of enals (r=0.935; 0.065), ketones (r=0.935; 0.065), branched alkanes (r=0.936; 0.064), and alcohols (r=0.945; 0.055) with chicken fat aroma approached significance at noted levels. Likewise, correlations of concentrations of aldehydes (r=0.925; 0.075), alkanes (r=0.936; 0.064), and aromatic hydrocarbons (r=0.928; 0.072) with chicken fat flavor approached significance. Lack of correlation between aldehydes, long considered indicators of oxidation, and oxidized aroma and flavor notes may be attributable to the large variation between replicates for aldehyde concentration, perhaps due to initial differences in fatty acid profiles for replicate fat sources.

Fifteen individual identified compounds consisting mainly of branched alkanes and aldehydes correlated with chicken fat aroma (Table 4) as did an additional 22 unknown compounds. Noted compounds of interest with correlations between concentrations and chicken fat aroma bordering on significance included 2-hexenal (r=0.926; 0.074), 2(E)-heptenal (r=0.920; 0.080), octanal (r=0.944; 0.056), nonanal (r=0.940; 0.060), 2(E)-nonenal (r=0.926; 0.074), and 2,4-nonadienal (r=0.913; 0.087). Chicken fat flavor correlated with 9 individual identified compounds (Table 4) of which 5 previously correlated with chicken fat aroma. These volatiles consisted mainly of branched alkanes and alkenes. An additional 8 unknown compounds correlated with chicken fat flavor, 7 of which previously correlated with chicken fat aroma. Pentanal (r=0.939; 0.061), hexanal (r=0.947; 0.053), and 2,6-di-*t*-butyl-4-methylphenol (r=0.920; 0.080) concentrations approached significant correlations with chicken fat flavor. A similar trend occurred for oxidized and chicken fat notes and correlation between compounds like pentanal and hexanal, which have correlated with oxidized notes (Evans et al., 1971), with chicken fat aroma and flavor. This indicated that oxidized sensations, within a range of acceptability, may be important in chicken-specific sensory attributes, and the descriptors may not be entirely separable by panelists.

Alkanes, ketones, and saturated alcohols from lipid oxidation are not very important to aroma and flavor (Forss, 1969). Aldehydes and related compounds are very important due to their higher concentrations and lower threshold values for perception and have been a primary focus for monitoring oxidation (Evans et al., 1971; Shahidi et al., 1986). All treatments yielded hexanal and decadienal concentrations in excess of some published flavor threshold values in similar media. However, most published thresholds were determined in single compound systems that did not consider possible synergies and masking of other components which may apply in a system with more than 300 quantitated volatiles.

In order to reduce the numerous correlations, specific attention was directed to correlations between volatile concentrations that differed by treatment (Table 2) and sensory attributes that differed by treatment. Of the 25 such identified compounds and 2 aroma notes the only correlations demonstrated were for concentrations of 4-methyloctane (r=0.971; 0.029); 2,2,4-trimethylheptane (r=0.987; 0.013); 2,2,6-trimethylheptane (r=0.985; 0.015); and 2-heptanone (r=0.977; 0.023) with chicken fat aroma. Lack of obvious correlations that were expected based on previous results for compounds like nonanal, 2(E)-nonenal, decanal, and 2(E)-undecenal was partially due to variation across replicates in fat source and extent of oxidation during cooking and extraction.

CONCLUSIONS

AROMA AND FLAVOR VOLATILES from cooked chicken fat, including classes of volatiles like aldehydes and enals that have been linked to species-specific notes were concentrated by supercritical fluid extraction with carbon dioxide. Concentration was most efficient at the lowest pressure, near the critical point. Sensory characteristics of extracts were affected by changes in volatile profiles. Similarities between identified volatiles correlated with chicken specific aroma and flavor, and published correlations for volatiles and oxidized notes, indicates that oxidized attributes were important in chicken flavor.

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Interactions Between Carnosine and the Different Redox States of Myoglobin

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ABSTRACT

To better understand the mechanism by which carnosine inhibits myoglobin oxidation in salted ground pork, interactions of carnosine with ferrylmyoglobin (ferMb), metmyoglobin (metMb) and oxymyoglobin (oxyMb) were investigated. Carnosine (0–50 mM; pH 5.0–7.5) accelerated the conversion of metMb to oxyMb at pH ≥ 7.0 and carnosine concentrations ≥ 25 mM. Carnosine (1–50 mM) also accelerated the conversion of oxyMb to metMb with its formation rates increasing with decreasing pH and increasing carnosine concentrations. Carnosine (1–25 mM) inhibited ferMb-catalyzed oxidation of phosphatidylcholine liposomes 16–76% and reduced the ferMb electron paramagnetic resonance signal 24–43%. Results suggested that the color stabilizing effects of carnosine were related to its antioxidant activity.

Key Words: carnosine, oxymyoglobin, metmyoglobin, lipid oxidation, meat

INTRODUCTION

OXIDATIVE REACTIONS in muscle foods lead to degradation of lipids and proteins resulting in deterioration of flavor, texture and color. Carnosine (N-β-alanyl-L-histidine) and anserine (N-β-alanyl-L-1-methyl-histidine) are dipeptides endogenous to skeletal muscle. Their concentrations range from 5–70 mM in beef, pork, chicken and fish (Crush, 1970; Plowman and Close, 1988). White muscle fibers generally have higher anserine and carnosine concentrations than red muscle. Combined dipeptide concentrations in chicken are 71 mM in breast and 12.2 mM in leg muscle (Crush, 1970).

Carnosine can inhibit lipid oxidation promoted by the major skeletal muscle oxidation catalysts (e.g., iron, copper, heme-containing proteins and lipoxygenase) at the pH and temperatures common to muscle foods (Boldyrev et al., 1988; Decker and Faraji, 1990; Decker et al., 1992). The antioxidant activity of carnosine is multifunctional, acting as both a metal chelator (Kohen et al., 1988; Decker et al., 1992) and a free radical scavenger (Dahl et al., 1988; Rubtsov et al., 1991; Chan et al., 1994). Carnosine (0.5–1.5%) inhibits lipid oxidation in pork (Decker and Crum, 1991; 1993), turkey (Calvert and Decker, 1992) and beef (Shantha et al., 1995). Addition of carnosine (0.5%) to frozen (−15°C), salted (2% NaCl), ground pork also inhibits oxidation of myoglobin. The color protecting effects of carnosine in salted ground pork were greater than butylated hydroxytoluene (BHT), tocopherol, rosemary extract or sodium tripolyphosphate (Decker and Crum, 1991; Decker et al., 1993).

Research has been reported on the ability of carnosine to interact with prooxidant metals and free radicals (Kohen et al., 1988; Decker et al., 1992; Dahl et al., 1988; Rubtsov et al., 1991; Chan et al., 1994). However, little work has been reported on the ability of carnosine to interact with myoglobin. If carnosine can stabilize oxymyoglobin (Fe^{II}) or reduce metmyoglobin (Fe^{III}), then its presence could influence the quality and shelf-life of muscle foods by decreasing meat discoloration. However, the oxidative state of myoglobin is also influenced by lipid oxidation reactions (Yin and Faustman, 1993; Govindarajan et al., 1977; Lin and Hultin, 1977). Therefore, carnosine's color stabilizing properties could be related to its interactions with prooxidants and free radicals (Chan et al., 1994; Decker et al., 1992; Kohen et al., 1988; Decker and Faraji, 1990). Interactions between myoglobin and hydrogen peroxide yield ferrylmyoglobin radicals which contain Fe^{IV} and a free radical associated with tyrosine residues on the protein (Giulivi et al., 1992). Ferrylmyoglobin, an active lipid oxidation catalyst (Harel and Kanner, 1985), can be inhibited by carnosine (Decker and Faraji, 1990), however, the basis of this inhibition is not understood.

Our objective was to better understand the mechanisms by which carnosine inhibits oxidation of lipids and myoglobin in salted ground pork by studying interactions between carnosine and different redox states of myoglobin.

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MATERIALS & METHODS

CARNOSINE, hydrogen peroxide (30%), ethylene diaminetetraacetic acid (EDTA), soybean phosphatidylcholine (Type IV-S) and metmyoglobin (horse heart muscle, Type I) were obtained from Sigma Chemical Co. (St. Louis, MO). The spin trap 5,5'-dimethylpyrroline-N-oxide (DMPO) and 4,6-dihydroxy-2-mercaptopyrimidine (thiobarbituric acid; TBA) were from Aldrich Chemical Co. (Milwaukee, WI). DMPO radical adducts were not observed in commercially prepared DMPO. Therefore, no further purification was performed. The mixed bed ion exchange resin, AG 501-X8, was obtained from Bio-Rad (Hercules, CA). All other chemicals were reagent grade or purer.

Myoglobin preparation

Oxymyoglobin (oxyMb) was prepared by the reduction of metmyoglobin (metMb) with sodium hydrosulfite. Both oxyMb and metMb were passed through a 1 × 40 cm column of Bio-Rad Ag 501-X8 mixed bed ion exchange resin before use to remove hydrosulfite breakdown products and free iron (Brown and Mebine, 1969). Ferrylmyoglobin (ferMb) was generated using metmyoglobin (400 μM) and hydrogen peroxide (400 μM) in 0.1M phosphate buffer (pH 7.4) containing 1 mM EDTA (Xu et al., 1990). The mixture was allowed to react for 1 min followed by desalting through a Sephadex G-25 column (2 × 20 cm) at a flow

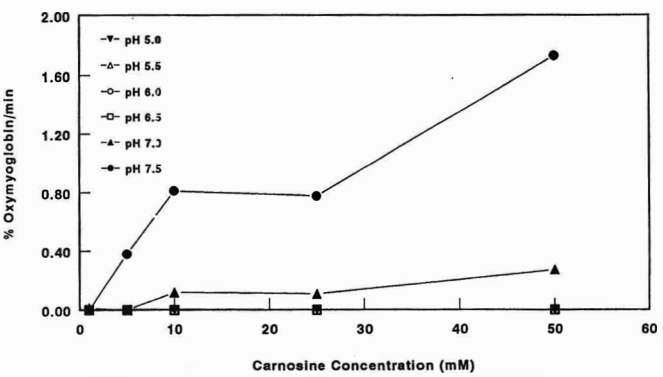

Fig. 1—Conversion of metmyoglobin to oxymyoglobin as related to pH and carnosine concentrations. Incubation at 25°C for 20 min.

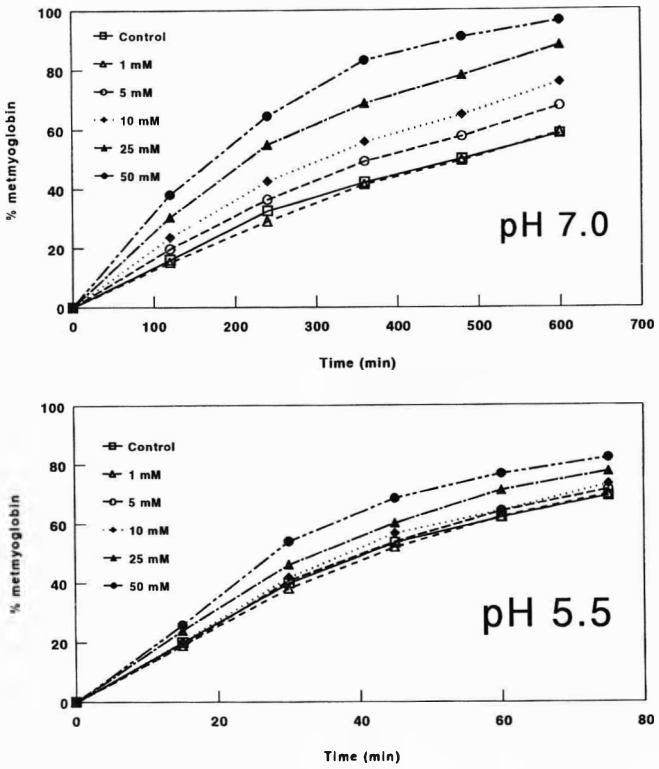

Fig. 2—Conversion of oxymyoglobin to metmyoglobin at 25°C in the presence of carnosine (0–50 mM) at pH 7.0 and 5.5.

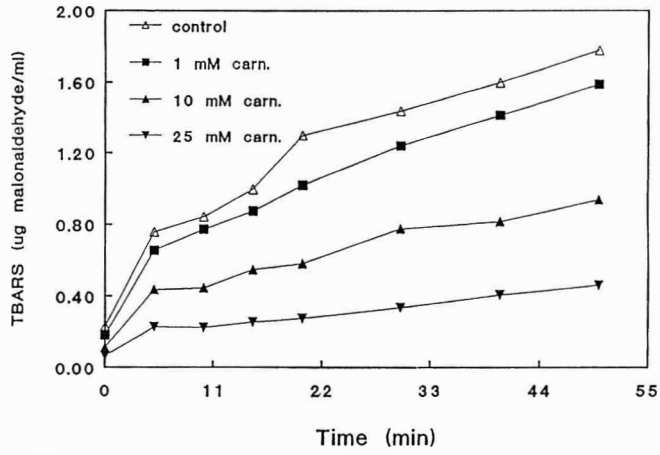

Fig. 3—Catalysis of phosphatidylcholine liposomes oxidation by ferrylmyoglobin (40 μM) as related to carnosine concentration. Lipid oxidation based on TBARS.

rate of 5 mL/min using 0.1M phosphate buffer (pH 7.4) to separate ferrylmyoglobin from unreacted hydrogen peroxide and EDTA. All analyses of ferryl myoglobin were completed within 10 min after reacting with hydrogen peroxide. Spectrophotometric analysis of myoglobin derivatives were conducted on a Shimadzu 2101 UV/visible spectrophotometer at room temperature (≈23°C). Absorbance scans (450–650 nm; 1600 nm/min) were performed on 40 μM ferMb or 70 μM metMb and OxyMb in 0.1M phosphate buffer over the pH range 5.0–7.0 in the presence and absence of carnosine (1–50 mM). OxyMb and metMb concentrations were determined spectrophotometrically with absorbance values at 572, 565, 545 and 525 nm (Krzywicki, 1982). Ferrylmyoglobin concentrations were calculated using the formula [ferMb] (μM) = 249 $Abs_{550\ nm}$–367 $Abs_{630\ nm}$ (Giulivi et al., 1992).

Electron paramagnetic magnetic (EPR) measurement

Desalted ferrylmyoglobin (400 μM) was immediately mixed with DMPO (10 or 100 mM) and carnosine (0, 1, 10, or 25 mM) and transferred to a quartz flat cell which was centered in a TM EPR resonant cavity for analysis. Spectra were recorded at room temperature using a Bruker 300 EPR spectrometer with computerized data acquisition and analysis. Conditions for the spectrometer were 7.45 mW microwave power, 0.98 Gauss (G) modulation amplitude, 75 G scan width, 14.3 G/sec scan rate, 1.28 ms time constant and 9.79 GHz microwave frequency.

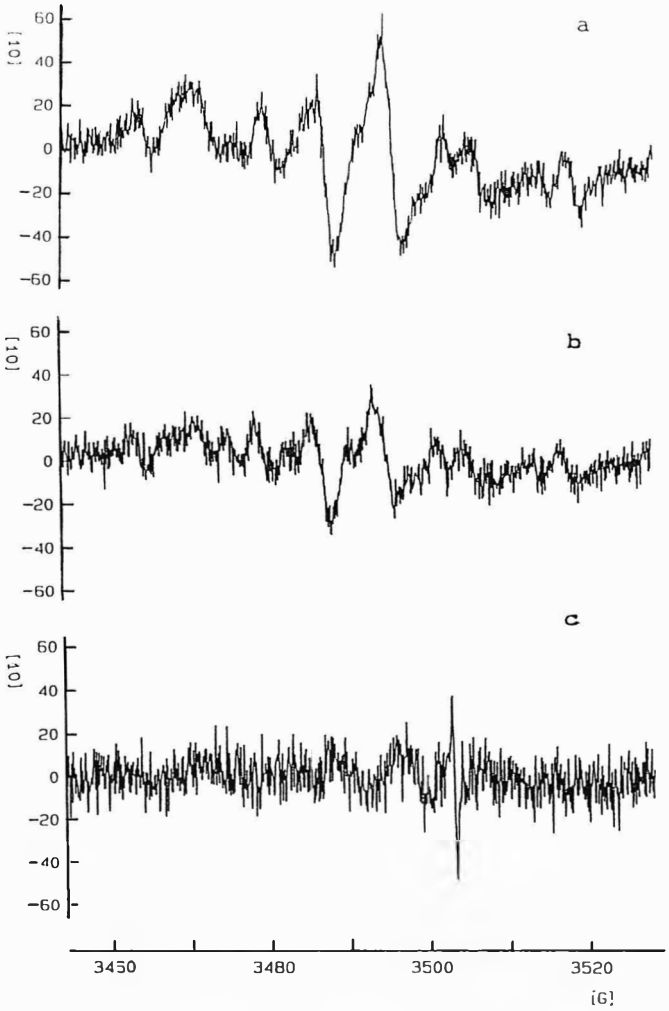

Fig. 4—Electron paramagnetic resonance spectra of: (a) ferrylmyoglobin (40 μM) + DMPO (100 mM); (b) ferrylmyoglobin (40 μM) + DMPO (100 mM) + carnosine (25 mM); and (c) ferrylmyoglobin (40 μM) + DMPO (10 mM) + carnosine (25 mM).

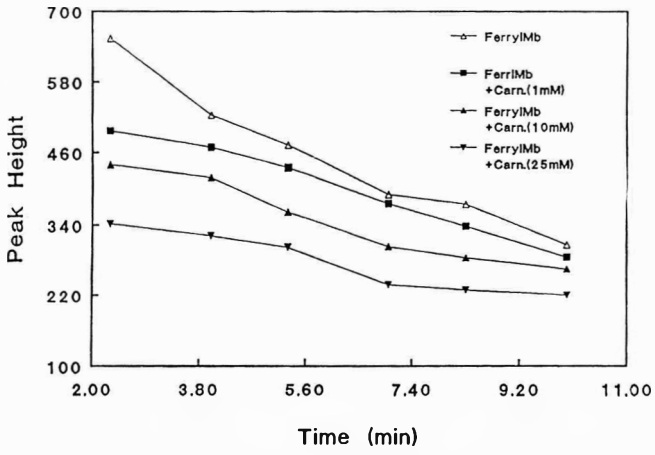

Fig. 5—Reduction of the ferrylmyoglobin electron paramagnetic resonance signal (3483 Gauss) as related to concentration of carnosine.

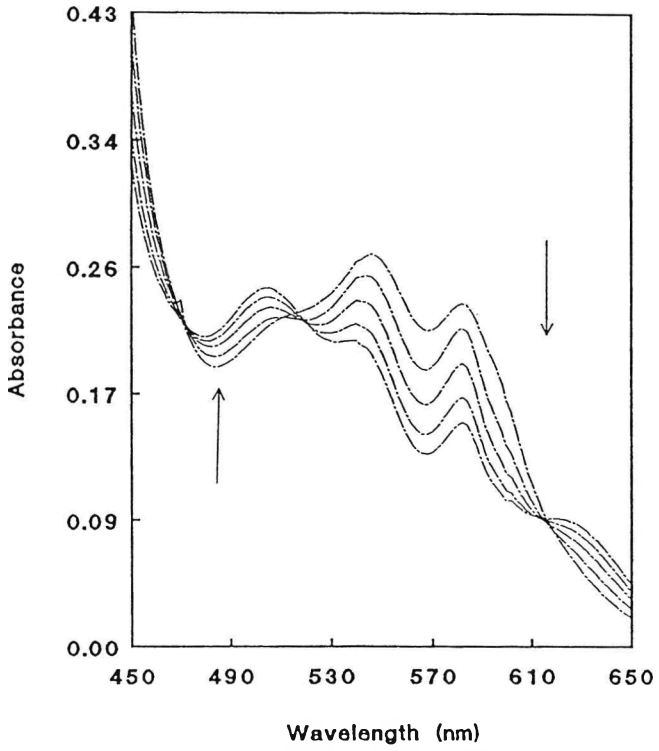

Fig. 6—Changes in absorbance of ferrylmyoglobin in the presence of 25 mM carnosine at 25°C and pH 7.4. Each line represents spectra at 2 min intervals. Arrows indicate direction of spectral shifts.

Lipid oxidation assay

The ability of carnosine (0–25 mM) to inhibit ferMb (40 μM)-catalyzed lipid oxidation was determined in a model system containing 0.02 mg phosphatidylcholine liposomes in 0.1M phosphate buffer (pH 7.4; Decker and Hultin, 1990). The extent of lipid oxidation in the model system was determined by periodically measuring the formation of thiobarbituric acid reactive substances (TBARS; McDonald and Hultin, 1987) during incubation in a shaking water bath (37°C) for up to 50 min.

RESULTS & DISCUSSION

CARNOSINE can maintain the red color of salted (2% NaCl), ground pork during frozen (−15°C) storage (Decker and Crum, 1991). The preservation of color could be due to stabilization of oxymyoglobin and/or reduction of metmyoglobin. Carnosine can interact with different redox states of myoglobin. At pH 7.0, carnosine concentrations common to muscle (5–25 mM) accelerated the conversion of metMb (Fe^{III}) to oxyMb (Fe^{II}) (Fig. 1) and oxyMb to metMb (Fig. 2a) at rates ranging from 0–0.11 and 0.11–0.17 μM/min, respectively. However, at a pH more common to muscle foods (5.5), carnosine (5–25 mM) did not catalyze conversion of metMb to oxyMb but accelerated the conversion of oxyMb to metMb from 0.9–1.1 μM/min (Fig. 2b). The observed conversion of oxyMb to metMb *in vitro* at a pH common to muscle foods was in contradiction to reported color stabilizing effects of carnosine in salted ground pork (Decker and Crum, 1991). This suggests that carnosine did not preserve color through direct interactions with myoglobin.

Oxidation of myoglobin can also be accelerated by lipid oxidative reactions (Yin and Faustman, 1993; Govindarajan et al., 1977; Lin and Hultin, 1977). Carnosine effectively inhibits iron- and copper-catalyzed lipid oxidation under conditions expected in muscle foods (Chan et al., 1994; Decker et al., 1992; Decker and Faraji, 1990). In addition to transition metals, the ferrylmyoglobin radical formed by activation of metMb by hydrogen peroxide is hypothesized to be an active lipid oxidation catalyst in muscle foods (Harel and Kanner, 1985). Carnosine (1–25 mM) inhibited the oxidation of phosphatidylcholine liposomes by ferMb 16–76% (Fig. 3) based on formation of TBARS.

Electron paramagnetic resonance spectroscopy was also used to study interactions between carnosine and ferMb. The observed DMPO-ferMb EPR spectrum (Fig. 4a) had resonance peaks at 3468 G, 3477 G, 3483 G and 3502 G peaks, similar to the DMPO-ferMb spectrum reported by Xu et al. (1990). Interactions between ferMb and Trolox (a water-soluble analog of α-tocopherol), glutathione and cysteine result in the formation of phenoxyl (Giulivi et al., 1992) and DMPO-thiyl radical adducts (Romero et al., 1992). Carnosine-DMPO radical adducts were reported in a hydroxyl radical generating system consisting of hydrogen peroxide and iron (Chan et al., 1994); however, carnosine-based radicals were not detected in the presence of ferMb, carnosine (25 mM) and 100 (Fig. 4b) or 10 mM DMPO (Fig. 4c). Carnosine (1–25 mM) reduced the ferMb EPR signal at 3483 G 24–48% after 2.5 min of incubation (Fig. 5). Peak intensity at 3483 G of the ferMb-DMPO radical adduct in all treatments decreased over a 10-min period. All decomposition rates were similar after 4 min.

Absorbance spectra (450–650 nm) of ferMb in the presence and absence of 25 mM carnosine (pH 7.4) were compared (Fig. 6). In the absence of carnosine spectra were similar to the ferMb spectra reported by Romero et al. (1992) and Kanner (1992) with peaks at 548 and 582 nm. Addition of carnosine resulted in conversion of ferMb to metmyoglobin as evidenced by the appearance of peaks at 504 and 630 nm. Conversion of ferMb to metMb by carnosine proceeded through a one electron reduction in a manner similar to cysteine and glutathione. This can be compared to dihydrolipoate which reduces ferMb to oxyMb via a two electron reduction (Romero et al., 1992). The ability of carnosine (25 mM) to reduce ferMb (4.2 μM ferMb converted to metMb/min; pH=7.4; Fig. 6) was 7.7-fold greater than its ability to reduce metMb (0.54 μM metMb converted to oxyMb/min; pH=7.5; Fig. 1).

CONCLUSIONS

CARNOSINE did not stabilize oxymyoglobin or significantly catalyze the reduction of metmyoglobin at the pH and carnosine concentrations found in skeletal muscle. Since carnosine inhibited myoglobin oxidation in salted, ground pork where lipid oxidation reactions were also prevalent, this suggested that its prevention of meat discoloration was related to its antioxidant properties.

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Ms received 6/23/95; revised 8/3/95; accepted 8/8/95.

This research was partially supported by the Pork Industry Group of the National Livestock & Meat Board.

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Ms received 12/9/94; revised 6/21/95; accepted 6/28/95.

Paper number FSR94-49 of the Journal Series of the Dept. of Food Science, Raleigh, NC 27695-7624. Use of trade names in this publication does not imply endorsement by the North Carolina State Agricultural Research Service, nor criticism of similar ones not mentioned. This material is based upon work supported under a National Science Foundation Graduate Research Fellowship and funding from the Southeastern Poultry and Egg Association.

Solvent Desorption Dynamic Headspace Method for Diacetyl and Acetoin in Buttermilk

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ABSTRACT

Major buttermilk volatiles recovered included diacetyl, acetic acid, and acetoin. Detection limits were diacetyl 0.2 and acetoin 2.0 μg/g. Mean percent recoveries were 112% for diacetyl and 8.08% for acetoin. Normalized detector responses were linear over the range of concentrations tested ($R^2 > 0.999$) for diacetyl and acetoin. Percent relative standard deviations from quadruplicate analysis of 7-day-old buttermilk were <8% for diacetyl, acetoin, and acetic acid. The method enabled quantitative estimation of diacetyl and acetoin in <30 min, including sample preparation time.

Key Words: dynamic headspace, buttermilk, diacetyl, acetoin, gas chromatography

INTRODUCTION

FERMENTATION and flavor development in buttermilk occur through the action of a mixed starter culture that contains both acid- and aroma-producing bacteria. The aroma-producing bacteria are responsible for fermentation of citrate into diacetyl which imparts the desirable buttery aroma. Diacetyl is reduced in the presence of diacetyl reductase into acetoin (Collins, 1971), resulting in a substantial loss of desirable aroma. The presence and quantity of acetoin is indicative of diacetyl reductase which can be found in starter cultures (Kneifel et al., 1992) and is commonly associated with psychrotrophic bacteria (Seitz et al., 1963).

The assessment of diacetyl, acetoin, and other compounds associated with the flavor and aroma of commercial buttermilk has been the subject of many research reports. Typical assays include colorimetry (Westerfeld, 1945; Pack et al., 1964; Walsh and Cogan, 1974), chemical derivatization (Damiani and Burini, 1988; Martineau et al., 1994), solvent extraction (Jansen et al., 1979), static headspace sampling (Ulberth, 1991), and thermal desorption dynamic headspace (TDD) sampling (Yang and Min, 1994). Each method has inherent limitations.

Static headspace sampling typically is not sensitive enough to directly assay for acetoin (Marsili, 1981; Ulberth, 1991). Thermal desorption dynamic headspace analysis can of detect many volatile compounds including diacetyl and acetoin (Laye et al., 1993). However, the high cost of thermal desorption and cryogenic focusing equipment has been prohibitive. Also, storage of static and dynamic headspace samples is impossible since the entire sample is injected at one time. Most of the methods involve heating, which can promote spontaneous breakdown of α-acetolactic acid into diacetyl (Jönsson and Pettersson, 1977), resulting in overestimation of diacetyl.

Solvent desorption dynamic (SDD) headspace sampling is a simple technique commonly employed for many volatile compounds (Olafsdottir et al., 1985; Rizzolo et al., 1992). However, few reports have been made concerning SDD to determine aroma compounds in fermented dairy products (Kang et al., 1988).

Our objective was to characterize the effectiveness of a low cost, commercially available solvent desorption trap to quantitatively measure diacetyl and acetoin in buttermilk.

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MATERIALS & METHODS

Model system

The ability of the SDD method to detect and quantify diacetyl and acetoin was examined with a fermented milk model system similar to that of Ulberth (1991). The model system consisted of commercially pasteurized, homogenized milk (2% fat) with varying concentrations of added diacetyl (0–32 μg/g) and acetoin (0–512 μg/g) standards. Lactic acid (J.T. Baker Inc., Phillipsburg, NJ) was added to simulate the pH (4.5) and provide the consistency of a fermented milk product. Diacetyl, acetoin, 2-butanone, acetic acid and 1-propanol were purchased from Sigma-Aldrich (St. Louis, MO). Standard purity was determined with preliminary gas chromatographic (GC) analysis. The model system was used to establish retention times of target compounds, develop calibration curves, determine % recovery and % relative standard deviation (RSD) and to estimate limits of detection (LOD) for diacetyl and acetoin.

Buttermilk samples

Containers (946 mL) of fiberboard-packaged buttermilk were obtained from Lochmead Dairy (Junction City, OR) on the day of manufacture and stored at 4°C. Duplicate cartons were randomly selected for sampling at 3-day intervals. RSDs for quantifiable aroma compounds were determined by quadruplicate analysis of buttermilk samples at 7 days storage.

Dynamic headspace analysis

A 10-g sample was placed in a 40-mL glass vial and sealed with a screw-top Teflon-lined cap. A 100-μL aliquot of 1-propanol internal standard solution (5 μg/g) was added to each vial prior to homogenization with a vortex mixer. A 3-g sample was transferred from the glass vial into a 5-mL round-bottom flask with a universal inlet adapter.

Sample purge was conducted by immersing the flask in a 30 ± 1°C circulating water bath. A submersible stirrer on highest setting and Teflon starburst stirring head (9.5 mm, Fisher Scientific, Pittsburgh, PA) were used to provide thorough agitation of samples, facilitate heat transfer, and increase gas/liquid interaction. Nitrogen was the purge gas to limit oxidative changes (Monnet et al., 1994). Purge gas was passed through a moisture and hydrocarbon trap (Restek, Bellefonte, PA) . Teflon tubing was used for all connections following the gas filters. Purge was conducted by swept surface (Kang et al., 1988) to avoid foaming and inclusion of antifoam agents. Purge volume (V_a) was estimated for 99% recovery (P) of diacetyl using the relationship

$$V_a = -V_w K^{-1} \ln[(100 - P)/100]$$

where K is the air/water partition coefficient of diacetyl at 25°C and V_w is the volume of aqueous solution (Buttery et al., 1987). The K for diacetyl was computed using the equation (Buttery et al., 1971)

$$K = p/N \times 0.97 \times 10^{-6}$$

where p is the vapor pressure of pure diacetyl at 25°C (Lidl, 1991) and N is the solubility in water expressed as a mole fraction (Thanh et al., 1992). Thus,

$$K = 55.88/0.5233 \times 0.97 \times 10^{-6} = 1.036 \times 10^{-3}$$

A rate of 800 mL/min was the minimum purge rate to prevent visible condensation of water in the adsorbent bed. The final calculated purge volume was 16L.

Commercially available Carboxen traps (ORBO 90, Supelco, Bellefonte, PA) were used because they trap carbonyl compounds are hydrophobic, and are relatively chemically inert (Betz et al., 1989). Following sample purge, traps were immediately eluted with GC/MS grade acetone (Sigma-Aldrich, St. Louis, MO). The first mL of solvent eluate was

Table 1—Precision and accuracy of diacetyl and acetoin estimation in fermented milk model system

Diacetyl				Acetoin				
Expected[a]	Found	%Recovery	%RSD[b]	Expected	Found	Predicted	%Recovery	%RSD
0	ND[c]	—	—	0	ND	—	—	—
1	1.16	115	3.00	1	ND	—	—	—
2	2.05	102	1.38	2	0.020	2.14	1.00	165
4	4.50	112	7.32	4	0.285	4.80	7.12	10.9
8	9.31	116	0.95	8	0.633	8.30	7.92	4.26
16	17.38	108	3.55	16	1.36	15.6	8.41	4.15
32	37.34	116	0.55	32	3.00	32.1	9.36	2.58
				64	6.15	63.8	9.61	9.53
				128	11.7	120	9.17	3.79
				256	26.4	270	10.3	5.26
				512	50.4	509	9.84	0.782

[a] µg/g.
[b] % relative standard deviation from triplicate analyses.
[c] ND = not detected.

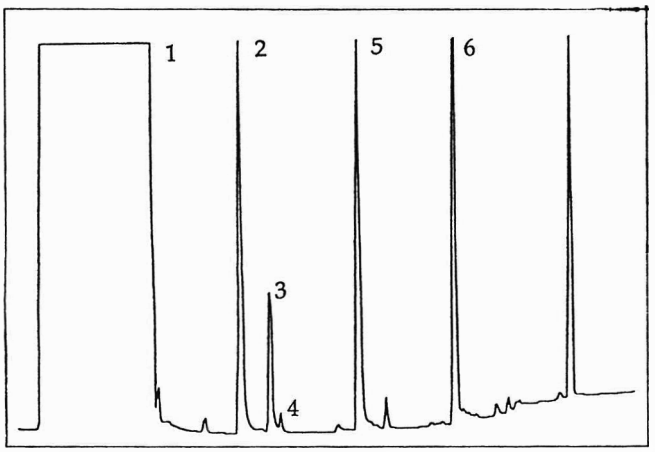

Fig. 1—Gas chromatogram of volatile compounds from buttermilk. Separations on a 6% cyanopropylphenyl, 94% methyl silicone fused silica capillary column (30 m × 0.32 mm i.d. × 1.8 µm film thickness). Unidentified peaks are solvent contaminants. (1) acetone; (2) 1-propanol; (3) diacewtyl; (4) 2-butanone; (5) acetic acid; (6) acetoin.

collected in a 1.0-mL volumetric flask. Eluting with this volume resulted in complete desorption of diacetyl and acetoin from the sorbent material. The eluate was then transferred to a 2-mL glass vial with Teflon-lined cap. A new trap was used for each analysis. Traps were examined for analyte bleed by placing the breakthrough section of the trap in a 2-mL glass vial, eluting with 100 µL of acetone, and conducting GC analysis for volatile compounds.

Separation and identification of volatiles

Identities of diacetyl, acetic acid and acetoin were determined by retention time of injected standards and were confirmed with mass spectroscopy. GC was carried out with a Hewlett Packard (HP, Avondale, PA) 5890 gas chromatograph with flame ionization detector (FID) under the following conditions. Initial temperature was 35°C isothermic for 7 min, increased at 20°C/min to 120°C, then increased at 40°C/min to 200°C and maintained for 5 min. A Restek (Bellefonte, PA) Rtx-624 column was employed to separate the compounds (30 m, 0.32 mm i.d., 1.8 µm film thickness). Column flow rate was 1.5 mL/min. Sample size was 1 µL, splitless injection. An HP 3396 integrator was used for peak area determination.

Mass spectroscopy was performed with a Varian 3400 (Palo Alto, CA) mass spectrometer with column and chromatographic conditions as described. Conditions were ion source temperature, 170°C; ionization voltage 70 eV; mass scan range, m/e 20–200; scan rate, 1.0 scan/sec. Compounds were tentatively identified in buttermilk by comparison of sample spectra with library reference spectra and further confirmed by comparing mass spectra and GC retention times to known standards.

RESULTS & DISCUSSION

Method development

It is possible to adjust method parameters to improve sensitivity. A target LOD of 0.2 µg/g for diacetyl was established since it is the sensory threshold of diacetyl in acidified milk (Bennett et al., 1964). The LOD for acetoin was 2.0 µg/g. Initially, solvent desorption was performed by placing the purge tube sorbent into a 2-mL vial followed by addition of 300 µL CS_2. However, upon storage diacetyl levels decreased by 40%. Analysis of samples with markedly decreased levels of diacetyl showed no changes in levels of other analytes and no potential diacetyl breakdown products. Diacetyl loss was minimized by eluting the trap with solvent and collecting the sample in the absence of sorbent material.

Although CS_2 is typically the solvent used for SDD, acetoin is relatively insoluble in CS_2. Acetone was used because it dissolves both diacetyl and acetoin, is stabile, safe for handling, and availabile in relatively pure form. Although the acetone we used had been glass distilled by the manufacturer, several contaminants were detected. Since these had different retention times and did not interfere with target compound peaks, no attempts were made to further purify the acetone.

Model system analysis

Normalized FID response to added amounts of diacetyl and acetoin exhibited good linearity ($R^2>0.999$) over the concentrations tested. As predicted, recovery of diacetyl was high (Table 1). Continued sampling ($V_a>16$ L) resulted in no increased diacetyl levels. Recovery of acetoin averaged <10% for all concentrations tested. Although low recovery has been reported to result in poor reproducibility (Westerndorf, 1985), RSD's for acetoin were considered acceptable. Quantification of acetoin in unknown samples depends on calibration curves that correct for the % recovery or could be reported in relative amounts for treatment comparisons. These results would be expected given the relative difference in boiling points (88 vs 147°C) and air/water partition coefficients of diacetyl and acetoin.

Buttermilk analysis

Several compounds were identified in buttermilk samples (Fig. 1). Quantification of ethanol and acetaldehyde was impossible with the described purge parameters. Both coeluted with an acetone solvent contaminant peak and exhibited sorbent bed breakthrough. No breakthrough of diacetyl, acetoin, acetic acid, or 2-butanone was indicated.

Method precision for quantifiable aroma-contributing compounds in buttermilk was highest for diacetyl and least for acetic acid (Table 2). However, all RSD's were considered quite low and related well to reported values (Ulberth, 1991; Monnet et al., 1994).

Aroma profiles of buttermilk samples analyzed over 15 days storage at 4°C showed changes (Marsili, 1981; Kang et al., 1988; Monnet et al., 1994) typical of high-quality fermented milk (Fig. 2). All compounds reached maximum levels at 9–12 days storage and then diminished in concentration.

Table 2—Gas chromatographic detection of buttermilk aroma volatiles

Compound	RT[a]	RSD[b]
Acetone	6.51	ND[d]
1-Propanol[c]	8.83	ND
Diacetyl	9.42	3.21
2-Butanone	9.61	ND
Acetic acid	11.13	7.08
Acetoin	12.75	6.36

[a] RT = retention time in min with Rtx-624 30m capillary column.
[b] %RSD = % relative standard deviation based on quadruplicate analysis of 7-day-old buttermilk samples.
[c] Internal standard.
[d] ND = not determined.

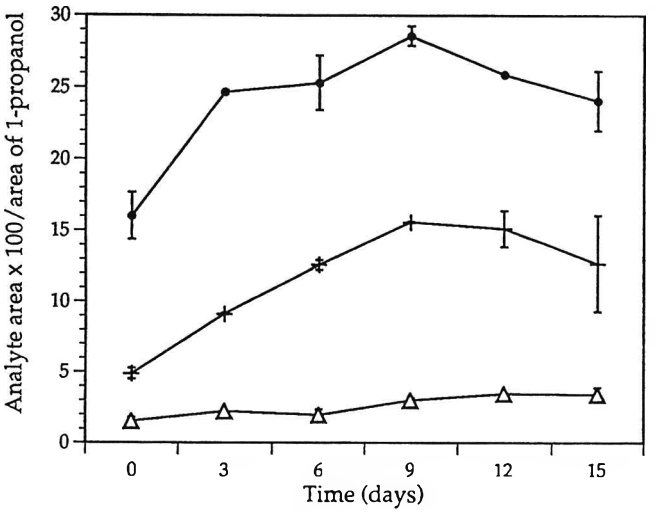

Fig. 2—Profiles of diacetyl (△), acetic acid (+), and acetoin (●) in buttermilk samples over 15 days storage at 4°C. Error bars represent standard deviations from duplicate analysis.

CONCLUSIONS

USING COMMERCIALLY AVAILABLE, low cost, preconditioned adsorbent traps resulted in a rapid, convenient method for estimating diacetyl and acetoin. Headspace sampling parameters and solvent choice could be changed to optimize recovery and quantification of other aroma compounds. This method is more effective than colorimetric tests commonly employed as it has less chemical interference and is capable of quantifying several volatile compounds simultaneously. Other advantages include minimal sample preparation, low heat treatment, modest equipment investment, and rapid sample analysis.

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Ms received 4/10/95; revised 7/18/95; accepted 7/22/95.

Technical Paper No. 10,724 from the Oregon State University Agricultural Experiment Station.

This research was supported with funds from the Eckelman Foundation, Oregon State University.

Appreciation is expressed to Lochmead Dairy (Junction City, OR) for the contribution of buttermilk samples.

Rheological Properties of Cheddar Cheese as Influenced by Fat Reduction and Ripening Time

Z. USTUNOL, K. KAWACHI, and J. STEFFE

ABSTRACT

Fat reduction in Cheddar cheese resulted in an increase in viscoelasticity as evidenced by increases in G' and G". Proteolysis during ripening led to softening of all cheeses and thus decreases in G' and G" for cheeses containing 34, 27, and 20% fat. Cheese with 13% fat showed a decrease in G' upon ripening, but no change in G". This lack of change in viscous behavior may be important to the texture of reduced-fat Cheddar cheese and overall acceptability. Dynamic rheological testing was helpful in understanding rheological behavior associated with fat reduction in cheese.

Key Words: Cheddar, cheese, dynamic rheology, proteolysis.

INTRODUCTION

MANY REDUCED-FAT dairy products were introduced in 1992 and 36% were cheeses (O'Donnell, 1993). Poor texture, slow flavor development and poor keeping quality have been common problems in reduced-fat cheeses. Cheese is viscoelastic in nature and exhibits both solid (elastic) and fluid (viscous) behavior. Cheese consists of a continuous protein matrix with a loose and open structure with dispersed fat globules. The nature of the network mostly determines rheological and fracture properties. As fat content decreases the protein matrix becomes more compact with less open spaces thus affecting texture (Bryant et al., 1994) and overall acceptability (Bryant, 1993). Increasing moisture is generally recommended to help improve texture of reduced-fat cheeses. Bryant et al. (1994) however reported that increase in moisture of reduced-fat Cheddar cheese did not improve texture attributes as determined by TPA.

Dynamic rheological testing has many applications in the food industry (Steffe, 1992; Rao and Steffe, 1992) and has been applied to cheese. Nolan et al. (1989) studied the dynamic rheological properties related to meltabilities of natural and imitation low-moisture part-skim Mozzarella. Measurements of viscoelastic moduli and dynamic viscosities helped provide basis for distinguishing between imitation and natural Mozzarella. Taneya et al. (1979) measured the dynamic properties of Gouda, Cheddar and process cheese. Tunick et al. (1990) examined viscoelastic properties of Cheddar and Cheshire cheeses. They suggested this method could be used in identification of the two cheeses to prevent mislabeling. Nolan et al. (1990) compared rheological behavior of Cheddar and pasteurized process American cheeses by dynamic rheological properties. The dynamic viscosity of both cheeses followed an Arrhenius-type relationship.

Our objective was to investigate the influence of milk fat reduction on rheological properties of Cheddar cheese using dynamic testing to provide fundamental information on rheology of reduced-fat Cheddar cheese. This should provide insight into the effects of composition and ripening on rheology and thus aid in development of reduced-fat cheese products.

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MATERIALS & METHODS

Cheese manufacture

Cheddar cheese with varying fat levels (34, 27, 20, and 13%) was manufactured from milk standardized to the appropriate fat levels. Standardized milk was warmed to 31°C and inoculated with 0.02% DVS (Direct Vat Set) culture (DVS #980, Chr. Hansen's Laboratory, Inc., Milwaukee, WI). Milk was ripened for 1 hr, and 0.01% chymosin (Chymax-Double strength, Pfizer, Milwaukee, WI) was added to clot the milk in 30 min. Cheddar cheese was manufactured by the standard procedure outlined by Kosikowski (1982). Curd was salted, hooped and pressed for 18 hr. Manufactured cheeses were vacuum-packaged the next day and ripened at 7°C for 3 mo.

Rheological behavior

A Rheometrics fluid spectrometer (8400 Rheometrics Inc., Piscataway, NJ) was used to characterize the rheological properties of all Cheddar cheese samples. The tests were performed at a constant frequency of 1 rad/sec and a constant strain of 0.1% in a parallel plate apparatus (2.5 cm radius). Cheese samples were cut at 5°C to obtain disks approximately 6 cm diam and 1 mm to 2 mm using a meat slicer (Model 512, Hobart Co., Troy OH) and a cookie cutter. The cheese samples were cut larger than the plate to allow for shrinkage caused by dehydration of the sample during the heating process. Sample temperature was controlled by a programmer (MPT-microprocessor, Neslab Instruments, Inc., Newington, NH) using silicon oil (polydimethyl siloxane, Dow Corning 200, Dow Corning Co., Midland, MI) as a medium. The temperature was raised from 25 to 75°C at 2°C/min during the test. Data included the storage modulus, G', and loss modulus, G", which are components of the loss tangent (or tan δ). These parameters are related: $\tan \delta = G''/G'$.

Water-soluble nitrogen

Water-soluble nitrogen extracts were prepared by the procedure of Kuchroo and Fox (1982) with minor modifications. The cheeses were grated and 20g cheese and 60g distilled water were homogenized for 5 min. The mixture was placed in a centrifuge tube, allowed to warm to 40°C for 1 hr and then centrifuged at 3000 × *g* for 30 min. Distilled water (20g) was added to the residue and the extraction procedure was repeated. First and second extracts were mixed together and filtered through #54 filter paper. Nitrogen content was determined by Kjeldahl procedure (Marshall, 1992) and reported as % N of total cheese nitrogen.

Statistical analysis

The cheese manufacturing was replicated four times in a randomized block design. The Microcomputer Statistical Program (MSTAT) (Michigan State Univ., E. Lansing, MI) was used for analysis of data and for appropriate comparisons which were made of fat levels at specified temperatures.

RESULTS & DISCUSSION

THE COMPOSITIONAL DATA as expected showed moisture and protein of the cheeses increased ($P<0.05$) when fat was removed. Moisture content (wet basis) of cheeses containing 34, 27, 20, and 13% fat were 38.5 ± 1.34, 40.8 ± 1.35, 40.8 ± 1.81, and 44.7 ± 2.16%, respectively. Protein contents were 22.3 ± 1.74, 27.9 ± 1.53, 32.7 ± 1.26, and 36.4 ± 1.06%, respectively.

Reduction of fat in Cheddar cheese and ripening affected viscoelasticity as demonstrated by the G', G" and tan δ curves (Figures 1 and 2). Storage modulus (G') represents solid-like or elastic character of a viscoelastic material. Although the G' of the cheeses were not different at the initial 30°C, as the temperature increased to 70°C, G' became distinctly different. The G' for all cheeses steadily decreased with increase in temperature; however, this decrease was sharper for higher-fat cheeses (34 and 27% fat) than lower-fat cheeses (20 and 13% fat). This indicated that the change in elastic behavior of Cheddar cheese was only slight upon heating for reduced-fat Cheddar cheese. Overall, G' increased ($P<0.05$) with decrease in fat content. The 34% cheese had the lowest G' and the 13% fat cheese had the highest G' (upon heating to 70°C) suggesting that reduction in fat resulted in an increase in elastic (or solid-like) character of Cheddar cheese (Fig. 1a).

The loss modulus (G") represents the viscous component of a viscoelastic material. Changes in G" of the cheeses during heating to 70°C followed the same trend as that of G' although magnitudes of changes were smaller. The G" of the higher-fat cheeses (34 and 27% fat) decreased steadily with increase in temperature, whereas G" of lower-fat cheeses remained constant through heating to 70°C. This indicates that reduced-fat cheeses do not undergo changes in viscous properties upon heating (Fig. 2b). At any given point, G' was greater than G" which indicated that the elastic component contributed more to viscoelasticity than did the viscous component. This behavior was expected for a viscoelastic solid (Rao and Steffe, 1992).

All cheeses showed an increase in tan δ with increase in temperature (Fig. 1c) indicating that the elastic component of Cheddar cheese decreased more steadily than its viscous properties. Overall, tan δ was higher for higher-fat cheeses (34 and 27% fat) than for lower-fat cheeses (20 and 13% fat). This supported our hypothesis that the changes in elastic properties contribute more significantly to Cheddar cheese rheology than do the viscous characteristics. Visser (1992) suggested that 'stiffness' of cheese was related to its fat content and that at high temperatures 'stiffness' of cheese decreased with amount of fat due to melting of milk fat (Visser, 1992). The degree of proteolysis in cheese also contributes to softening of cheese because breakdown products of casein are water-soluble and cannot contribute to the framework provided by the protein matrix. Percent water-soluble nitrogen for 34, 27, 20, and 13% fat cheeses were 5.5, 5.1, 4.4, and 4,1, respectively (Table 1). The cheeses were tested for changes in rheological properties and water-soluble nitrogen at 2 wk and 1 mo of ripening. Changes were not statistically significant. At 3 mo ripening significant changes were observed in rheological properties.

Cheddar cheese that had been ripened for 3 mo (Fig. 2) both G' and G" of all cheeses increased compared to 1-day-old cheeses as determined at 30°C; however, they decreased when cheeses were heated to 70°C. Tan δ remained the same. All four cheeses upon heating showed decrease ($P<0.05$) in G' compared to their 1-day-old counterpart indicating that ripening affected the elastic properties. This change in elastic behavior due to ripening was also evident in lower fat cheeses. Again G' increased with decrease in fat content: 34% fat cheese had the lowest G' and 13% fat cheese had the highest G' upon heating to 70°C (Fig. 2a). For the same cheeses that were 3 mo old, changes in G" upon heating showed similar trends except for the cheese containing 13% fat. In cheeses containing 34, 27, and 20% fat, G" decreased with increase in temperature and G" was lower than their 1-day-old counterparts. However, G" of cheese containing 13% fat remained constant through heating. This indicated that, unlike the cheese containing 20% fat which softened during ripening (as evidenced by decrease in G' and G"), only the elastic component (G') of the 13% fat cheese was affected by ripening. This cheese did not change in its viscous properties (G") upon ripening which was typically observed with the other cheeses (Fig. 2b). Proteolysis was higher ($P<0.05$) in higher-fat cheeses than in lower-fat cheeses that were ripened

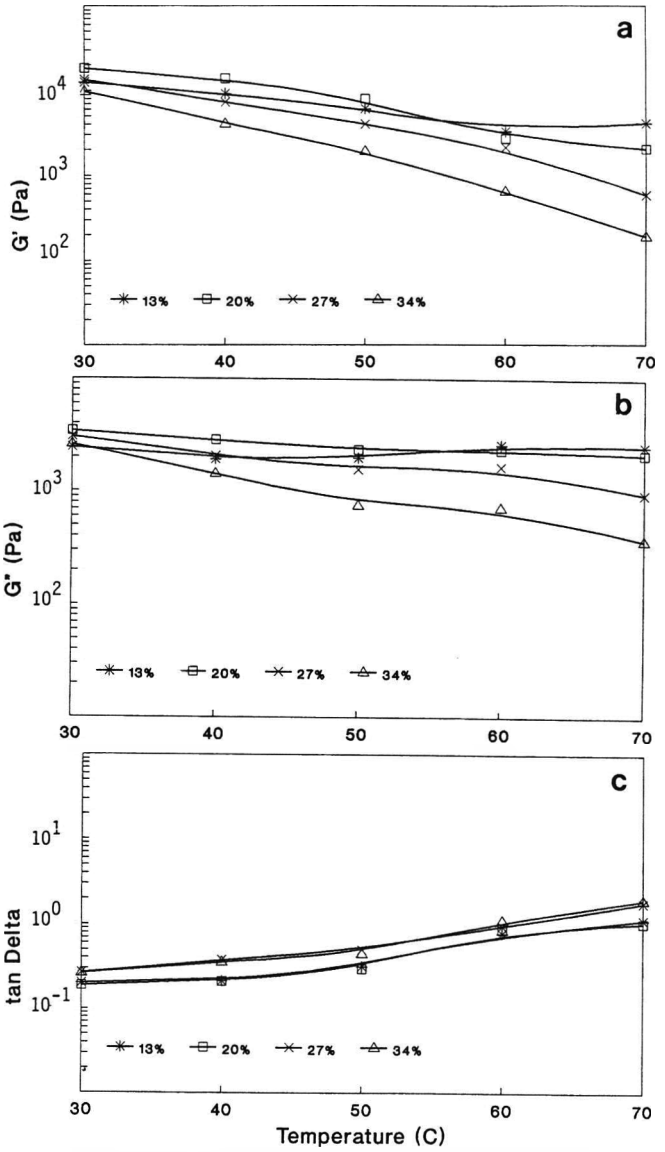

Fig. 1—Storage modulus (G'), loss modulus (G") and loss tangent (tan δ) of 1-day-old Cheddar cheese as influenced by fat content. Frequency, 1.0 rad/sec; strain, 0.1%. Each data point is generated by averaging four replications.

for 3 mo. Water-soluble nitrogen for 3-mo-old cheeses with 34, 27, 20, and 13% fat were 19.0, 17.6, 16.6, and 15.2%, respectively. This suggested that proteolysis during ripening contributed to the softening of the cheese and to the observed changes in viscoelastic behavior. For all cheeses ripened 3 mo, the tan δ curves were similar to 1-day-old cheese (Fig. 2c).

Bryant et al. (1994) investigated the impact of fat reduction in Cheddar cheese on microstructure by scanning electron microscopy (SEM), and perception of various texture attributes by TPA and a trained sensory panel. The panel reported that hardness and springiness of Cheddar cheese increased with decrease in fat content, whereas adhesiveness and cohesiveness decreased. Similar results were reported in Mozzarella cheese by Tunick et al. (1990). Bryant et al. (1994) showed (by SEM) that the loose open microstructure of the cheeses changed with reduction of fat and the protein network became dense and compact. Higher moisture content of the reduced-fat cheeses did not loosen the protein matrix or soften the cheese. However, increasing cheese moisture is recommended to cheese manufacturers to compensate for texture defects in reduced-fat cheeses. It has been hypothesized that cheese would become softer if its moisture content is elevated (Luyten, 1988). Our results were consistent with those of Bryant et al. (1994). Reduction in fat

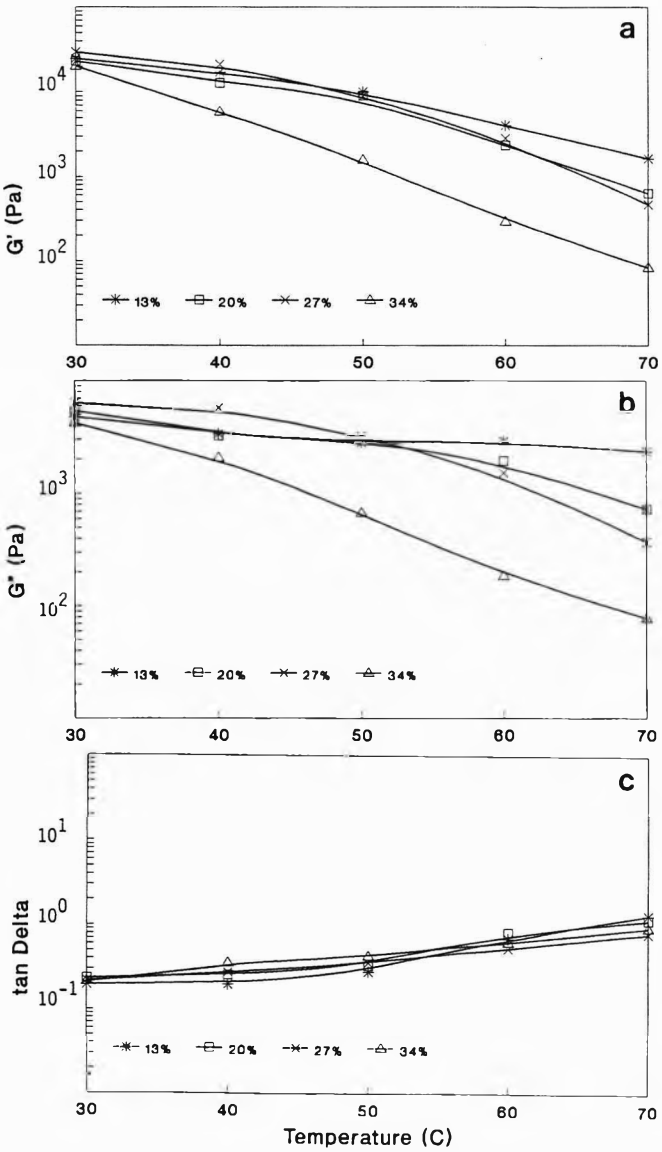

Fig. 2—Storage modulus (G'), loss modulus (G") and loss tangent (tan δ) of 3-month-old Cheddar cheese as influenced by fat content. Frequency, 1.0 rad/sec; strain, 0.1%. Each data point is generated by averaging four replications.

Table 1—Percent water-soluble nitrogen of Cheddar cheese as influenced by fat reduction and ripening time

Treatments[e] Cheese fat (%)	Ripening time	
	1 day	3 months
34	5.5[ab]	19.0[c]
27	5.1[ab]	17.6[cd]
20	4.4[a]	16.6[d]
13	4.1[a]	15.2[d]

[a-d] Means are reported as percentage nitrogen (of total cheese nitrogen). Means with the same superscript are not significantly different (P<0.05).
[e] n = 4 for all treatments.

resulted in an increase in both elastic and viscous properties, although these cheeses were higher in moisture than those with 34 and 27% fat. The higher moisture of the 13 and 20% fat cheeses did not contribute to softening. In our study, with exception of the cheese with 13% fat, only ripening resulted in softening of cheeses as evidenced by decrease of G' and G" curves. Proteolysis during ripening accounts for decreases in viscosity and elasticity of cheese (Creamer and Olson, 1982; Lawrence and Gilles, 1987). This effect was also demonstrated by Tunick et al. (1991) in Cheddar and Cheshire cheeses by downward shift in G' and G" curves.

Bryant (1993) reported that preference for Cheddar cheese decreased with decrease in fat content. Cheddar cheese with 21% fat was scored lower (P<0.05) in preference than those containing 34, 32 and 27% fat. Although the cheese with 21% fat was not rated unacceptable, it was not preferred. The cheese with 13% was scored unacceptable for texture. Although, this cheese had the highest % moisture, several panelists commented that it was 'waxy' and 'dry' (Bryant, 1993). It appears that the lack of change in viscous properties in reduced-fat Cheddar cheese may contribute to overall acceptability.

CONCLUSION

CHEDDAR CHEESE changed in rheological behavior as fat in the cheese was reduced and after 3 mo ripening. Fat reduction in Cheddar cheese resulted in an increase in viscoelasticity as evidenced by increase in G' and G". Proteolysis upon ripening provided for cheese softening as evidenced by a decrease in G' and G" for those containing 34, 27 and 20% fat. Although cheeses containing 13% fat showed a decrease in G' upon ripening, no change in G" was observed. This may be important to the texture of reduced-fat Cheddar cheese and overall acceptability.

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Ms received 12/15/94; revised 5/22/95; accepted 5/30/95.

Texture Evaluation of Ultrafiltered Teleme Cheese

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ABSTRACT

The texture of traditional and ultrafiltered (UF) teleme cheeses was evaluated using instrumental and sensory texture profile analysis. The UF teleme was made from unheated or heat treated (75°C for 5 minutes) UF milk concentrate. Samples were tested after 2 and 4 mo ripening. Instron variables did not discriminate between the three teleme types but sensory attributes, assessed by a panel discriminated the samples. Weak correlations were obtained between sensory and mechanical variables. Redundancy ordination analysis indicated that textural behavior of each type teleme cheese was characterized by different attributes. Sensory springiness or brittleness, lumpiness and fat content characterized the majority of traditional teleme samples whereas adhesiveness, pH, water soluble and noncasein nitrogen characterized the heat-treated UF samples.

Key Words: teleme cheese, texture profile, sensory discrimination

INTRODUCTION

TELEME CHEESE is a white brine cheese originated from the Balkans (Anyfantakis, 1991). It resembles feta cheese but differs in the way it is made. Teleme is manufactured almost entirely from cow's milk and its salting is in brine. Due to its easier and lower cost manufacture it became popular in Greece, where its consumption rivals feta.

Teleme cheese has never been commercially manufactured in Greece, using the ultrafiltration technique. Ultrafiltration is employed to increase cheese yield. Experimental manufacture of teleme using ultrafiltered (UF) milk concentrate showed that the yield was increased by as much as 40% in relation to traditional teleme (Antoniou et al., 1995). Also, feta cheese from UF milk concentrate differs in texture from that of the traditional feta (Lelievre and Lawrence, 1988). This is due to the incorporation of the whey proteins to the casein matrix. Moreover, the texture of feta cheese from heat-treated UF-milk concentrate is different from that from unheated UF-milk concentrate. This is attributed to the fact that in the heated concentrate the whey proteins are heat denatured and interact with the casein whereas in the unheated they simply act as inert filler in the case in matrix.

Little has been reported on the textural characteristics of teleme cheese. Our study was initiated to investigate the attributes which characterize the texture of teleme and to find out whether there are differences in textural behavior of the two types, traditional and UF-teleme cheeses. Texture is a sensory parameter which greatly affects consumer preference thus it is important to know whether differences in texture might exist between the two types which could affect overall acceptability.

Our objective was to evaluate texture employing both instrumental and sensory methods. The most commonly used method for assessment of food texture, the Texture Profile Analysis (TPA) technique (Szczesniak, 1966) was also applied. This method had been extensively employed for evaluation of texture of several varieties of cheese, albeit with various degrees of success (Lee et al., 1978; Chen et al., 1979; Fedrick and Dulley, 1984; Green et al., 1985; Jack et al., 1993).

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MATERIALS & METHODS

PASTEURIZED COW'S MILK standardized to 3.0% fat, purchased from a local dairy plant, was used for cheese making. Three types of teleme cheese were manufactured (a) according to the traditional method, (b) with unheated UF-milk concentrate, and (c) with heat-treated (75°C for 5 min) UF-milk concentrate.

The methods of preparing the three types have been reported (Antoniou et al., 1995). Three teleme manufacture runs were carried out within 18 mo. Samples were stored refrigerated for 4 mo and assessment of cheese texture was done after 2 and 4 mo of ripening (Table 1).

The ultrafiltration process was carried out using a batch type UF unit-module 20-2-LAB(DDS-RO-Division DK-4900 Nakskov Denmark). The module was a round plate type with membrane diameter 20 cm. The membranes used were of the GR 60 Type (DDS). The total membrane area was 0.36 m^2. The inlet temperature was 48—50°C and the operating pressure reached up to 8 bars before the desired maximum concentration factor (CF = 4.16) was achieved.

Composition

The chemical analysis of cheeses was carried out employing the following methods: Total solids content by the gravimetric method of Mumm et al. (1970); total nitrogen by the Kjeldahl method and fat content by the Gerber method (Kirk and Sawyer, 1991); salt content by the method of Scneider and Roeder (1979). The nitrogen content of the fractions nonprotein (NPN), water soluble (WSN) and noncaseinic (NCN) was determined by the Kjeldahl method and results were expressed as percentage of total nitrogen in the cheese.

Texture measurements

Textural properties were measured with an Instron Universal Testing Machine, Table Model 1140 (Instron Ltd, High Wycombe, Bucks, UK), operating in the compression mode. Four cylindrical samples were prepared from each cheese block and their dimensions were 23 mm both in diameter and height. Samples were cut at 4°C and left at room temperature (20°C) for at least 30 min prior to testing. Also, samples were taken from at least 5 mm deep in the cheeses, to limit the effects of surface drying (Jack et al., 1993). Each sample was compressed axially in two consecutive compression cycles by a 36 mm diameter flat plate probe attached to the moving crosshead. Testing conditions were: compression ratio 80% deformation from the initial height of the sample, crosshead speed 20 mm/min and chart speed 50 mm/min.

The textural parameters measured from the double curves were:

Hardness—the peak force during first compression cycle.

Fracturability—the force at first significant break in curve.

Table 1—Coding of teleme cheese samples and treatments

Samples	Manufacture run	Ripening period (months)	Code
Traditional	First	Two	TRA2a
"	Second	"	TRA2b
"	Third	"	TRA2c
UF-unheated	First	"	UFN2a
"	Second	"	UFN2b
UF-heat treated	First	"	UFH2a
"	Second	"	UFH2b
"	Third	"	UFH2c
Traditional	First	Four	TRA4a
"	Second	"	TRA4b
"	Third	"	TRA4c
UF-unheated	First	"	UFN4a
"	Second	"	UFN4b
UF-heat treated	First	"	UFH4a
"	Second	"	UFH4b
"	Third	"	UFH4c

Table 2—Mean percentage composition of two measurements for the teleme cheeses

Sample	DM[a]	Fat	TN	WSN/TN	NPN/TN	NCN/TN	Salt	pH
TRA2a	45.28	20.0	2.68	15.4	10.9	16.9	4.3	4.68
TRA2b	41.52	18.5	2.63	13.8	12.2	15.8	3.1	4.59
TRA2c	41.45	19.5	2.48	12.9	8.6	15.7	2.8	4.47
UFN2a	41.28	17.0	2.39	26.4	12.4	22.9	4.1	4.84
UFN2b	44.81	17.5	2.52	24.8	11.6	19.3	3.7	4.88
UFH2a	42.98	18.5	2.48	23.1	11.6	19.0	4.0	4.81
UFH2b	43.17	17.0	2.56	22.3	12.5	19.1	4.0	4.88
UFH2c	36.45	15.0	2.12	17.8	13.0	23.3	3.3	4.6
TRA4a	44.94	19.7	2.73	17.9	14.9	20.4	4.0	4.55
TRA4b	42.34	19.2	2.63	21.3	16.6	19.6	3.1	4.55
TRA4c	39.83	19.0	2.34	16.1	14.9	22.2	3.5	4.39
UFN4a	40.00	16.0	2.33	28.7	15.1	24.1	4.2	4.84
UFN4b	43.72	16.8	2.4	28.9	14.4	27.3	4.1	4.88
UFH4a	43.55	16.5	2.58	24.1	14.9	23.2	3.9	4.95
UFH4b	44.08	17.0	2.48	24.3	15.5	22.3	4.3	4.88
UFH4c	37.12	15.5	2.12	21.7	18.1	28.2	3.4	4.47

[a] Abbreviations are as follows: DM= dry matter, TN= total nitrogen, WSN= water soluble nitrogen, NPN= non proteinic nitrogen, NCN= non caseinic nitrogen.

Cohesiveness—the ratio of positive area during second compression to that during the first compression.

Springiness—the height that the food recovers during the time that elapsed between the end of the first compression cycle and the start of the second cycle.

Gumminess—the energy required to disintegrate a semi-solid food product to a state ready for swallowing; the product of hardness × cohesiveness.

Chewiness—the energy required to masticate a solid food product to a state ready for swallowing; the product of gumminess × springiness (Szczcesniak, 1966; Yang and Taranto, 1982).

Sensory evaluation

A panel of 11 members of the School's staff, 30 to 45 years of age, were selected on the basis of frequent consumption of cheese and cheese products, interest and availability. The panel members were given a brief initial training using commercial samples of different cheeses with characteristic texture: e.g. very soft, very hard, coarse etc, every time they were asked to participate in the sensory testing. They were trained to assess the following textural characteristics:

Hardness or firmness—force required to penetrate sample with molar teeth

Springiness or elasticity—bouncing property of sample through several consecutive bites

Adhesiveness—stickiness of sample in the mouth throughout mastication

Brittleness—breakability of sample at first bite

Cohesiveness—degree to which the cheese sample deforms before rupturing

Lumpiness—heterogeneous mouth-feeling of sample throughout mastication (Lee et al., 1978; Chen et al., 1979).

Each cheese sample was cut in 2 cm cubes. Samples were assessed in duplicate, at room temperature, and were presented to panelists in white plastic dishes. Order of assessment was randomized within sessions. Members were asked to evaluate the coded three different cheese samples (all of the same run and the same ripening period) for the intensity of the textural attributes, scoring on a 1 to 5 graduated scale assigned for each variable as follows: hardness—from very soft (1) to very firm (5); springiness—from very plastic (1) to very elastic (5); adhesiveness—from very slippery (1) to very sticky (5); brittleness—from very mushy (1) to very fragile (5); cohesiveness—from noncohesive (1) to very cohesive (5); lumpiness—from very smooth (1) to very grainy (5).

Statistical analysis

Data from composition measurements, Instron measurements and sensory analysis were subjected to 2-way ANOVA where the two treatments were the type of cheese and ripening period. Differences between means were detected by employing the Student-Newman-Keuls test for multiple comparisons (Zar, 1984). Redundancy analysis (Ter Braak, 1987; MacFie and Hedderley, 1993) was also carried out to determine (a) whether potential relations exist between the instrumental and sensory attributes, and (b) how these relationships are arranged and respond due to the influence of chemical composition of the cheeses, in order to adequately describe the texture.

The statistical packages used were Minitab and CANOCO (CANonical COrrelation Ordination) (Ter Braak, 1988).

RESULTS & DISCUSSION

WE COMPARED the composition (Table 2), the data obtained for instrumental parameters (Table 3) and the data from sensory analysis (Table 4) of the three types of teleme cheese. Note that data for the third batch of UF-non heat-treated teleme cheese were not included due to accidental faulty manufacture of the cheese which resulted in product failure.

The 2-way ANOVA (Table 5) shows that the UF-types of teleme contained less fat than the traditional teleme. These differences were due to the concentration factor (CF = 4.16) of the milk concentrate. The pH value of UF-cheeses was greater than that of the traditional teleme. The higher pH value in the UF-cheeses was due to the higher buffer capacity of the UF-milk concentrate in comparison to milk (Antoniou, 1986). The N-fractions; water soluble nitrogen/total nitrogen (WSN/TN) and noncasein nitrogen/total nitrogen (NCN/TN) were in greater concentrations in the UF-cheeses and their differences were significant compared to traditional cheese. This was due to the retention of whey proteins in both types of UF-teleme. For the nonprotein nitrogen/total nitrogen (NPN/TN) fraction there were no significant differences between the three types of teleme. During ultrafiltration the NPN content was the same in both milk and concentrate (Antoniou et al., 1995). The maturation period of the cheeses is important in the concentrations of all nitrogen fractions. Thus, all cheese types contained higher concentrations of nitrogen fractions after 4 mo ripening than after 2 mo. This increase was due to proteolysis.

Regarding the textural parameters, there were no significant differences between the three cheese types. The ripening period significantly affected hardness and gumminess, but all other attributes remained unaffected (Table 5). Both hardness and gumminess showed higher values in the 2-mo old cheeses than in the 4-mo old samples. This was because casein which is largely responsible for forming the cheese structure undergoes proteolysis resulting in weakening of the structure and softening of the cheese. The extent of proteolysis between the two ripening periods could be seen (Table 2). The same trend has been seen by other cheeses such as cheddar (Creamer and Olson, 1982), La Serena (Fernandez del Pozo et al., 1988), Cheshire (Tunick et al., 1990) and both traditional and UF-Gouda cheeses (Spangler et al., 1990). Since gumminess expresses the energy required to masticate the cheese and its value is dependent on hardness and cohesiveness, it obviously should be greater in the younger cheeses.

Sensory parameters showed statistically significant differences between the three types of teleme cheese whereas the ripening period did not have any effect (Table 5). The apparent discrepancy between results of the mechanical and sensory par-

Table 3—Mean values and standard deviations of four measurements for the textural parameters of teleme cheese determined by the Instron Universal Testing Machine

Sample	Hardness (N)	Cohesiveness	Springiness (m)	Gumminess (N)	Chewiness (Joule)	Fracturability (N)
TRA2a	27.1 ± 2.6	0.21 ± 0.04	0.004 ± 0.0	5.5 ± 0.26	0.02 ± 0.001	6.0 ± 2.0
TRA2b	37.5 ± 4.0	0.17 ± 0.02	0.005 ± 0.002	6.0 ± 1.16	0.06 ± 0.04	19.2 ± 4.0
TRA2c	24.0 ± 0.5	0.17 ± 0.04	0.006 ± 0.0	4.0 ± 0.6	0.02 ± 0.0	14.5 ± 0.3
UFN2a	19.7 ± 5.1	0.17 ± 0.02	0.004 ± 0.001	3.4 ± 1.7	0.01 ± 0.006	8.2 ± 2.3
UFN2b	36.2 ± 4.2	0.19 ± 0.04	0.007 ± 0.001	6.7 ± 0 5	0.05 ± 0.01	19.5 ± 5.2
UFH2a	27.2 ± 4.2	0.16 ± 0.05	0.003 ± 0.0	4.8 ± 2 2	0.01 ± 0.008	5.6 ± 0.7
UFH2b	16.4 ± 1.5	0.15 ± 0.02	0.004 ± 0.001	2.4 ± 0 1	0.01 ± 0.001	6.4 ± 0.0
UFH2c	26.5 ± 2.0	0.14 ± 0.0	0.003 ± 0.0	4.3 ± 0 5	0.01 ± 0.0	14.2 ± 0.5
TRA4a	32.0 ± 5.0	0.14 ± 0.01	0.005 ± 0.001	5.1 ± 2 4	0.03 ± 0.02	9.8 ± 2.8
TRA4b	23.0 ± 3.0	0.24 ± 0.0	0.004 ± 0.0	5.0 ± 0.5	0.02 ± 0.0	8.7 ± 1.5
TRA4c	29.0 ± 5.0	0.12 ± 0.0	0.003 ± 0.001	3.3 ± 0.8	0.01 ± 0.0	11.5 ± 3.0
UFN4a	14.2 ± 3.2	0.12 ± 0.03	0.004 ± 0.01	1.7 ± 0.24	0.01 ± 0.0	5.1 ± 0.3
UFN4b	22.5 ± 3.5	0.15 ± 0.03	0.005 ± 0.0	3.9 ± 1.2	0.02 ± 0.0	13.7 ± 0.0
UFH4a	15.0 ± 1.8	0.11 ± 0.02	0.003 ± 0.0	1.5 ± 0.05	0.005 ± 0.0	5.7 ± 0.2
UFH4b	11.1 ± 1.1	0.12 ± 0.0	0.006 ± 0.0	1.4 ± 0.05	0.01 ± 0.0	7.4 ± 0.2
UFH4c	13.0 ± 2.0	0.16 ± 0.0	0.004 ± 0.0	2.1 ± 0.5	0.01 ± 0.0	4.6 ± 0.2

Table 4—Sensory assessment mean scores and standard deviations of eleven judges for the teleme cheese texture

Sample	Hardness	Springiness	Adhesiveness	Brittleness	Cohesiveness	Lumpiness
TRA2a	3.1 ± 0.3	2.9 ± 0.6	2.9 ± 0.6	3.3 ± 0.5	4.0 ± 0.5	3.8 ± 0.7
TRA2b	3.3 ± 0.6	3.3 ± 0.8	2.5 ± 0.5	3.3 ± 0.5	3.5 ± 0.5	2.7 ± 0.8
TRA2c	3.5 ± 0.7	2.8 ± 0.7	2.9 ± 0.8	3.4 ± 0.5	3.4 ± 0.8	3.4 ± 0.7
UFN2a	2.3 ± 0.7	3.0 ± 0.7	2.9 ± 0.6	3.0 ± 0.0	3.6 ± 0.7	2.0 ± 0.0
UFN2b	3.3 ± 0.6	3.2 ± 0.6	2.8 ± 0.6	3.3 ± 0.5	2.3 ± 0.5	2.3 ± 0.5
UFH2a	1.6 ± 0.5	2.1 ± 0.6	4.1 ± 0.6	2.4 ± 0.5	2.6 ± 0.7	2.0 ± 0.0
UFH2b	2.1 ± 0.5	2.6 ± 0.8	3.8 ± 0.8	2.6 ± 0.8	2.0 ± 0.6	2.0 ± 0.6
UFH2c	2.2 ± 0.8	2.3 ± 0.9	4.1 ± 0.8	2.3 ± 0.5	2.8 ± 0.7	1.7 ± 0.7
TRA4a	3.4 ± 0.5	3.6 ± 0.5	2.7 ± 0.5	3.7 ± 0.5	3.3 ± 0.8	4.1 ± 0.7
TRA4b	3.4 ± 0.5	3.5 ± 0.8	2.6 ± 0.5	3.4 ± 0.7	3.8 ± 0.7	3.6 ± 0.9
TRA4c	3.2 ± 0.8	3.1 ± 0.7	2.8 ± 0.8	3.5 ± 0.5	3.2 ± 0.8	3.8 ± 0.7
UFN4a	1.9 ± 0.3	2.8 ± 0.8	3.5 ± 0.7	2.6 ± 0.5	2.9 ± 0.7	2.2 ± 0.4
UFN4b	3.0 ± 0.7	3.3 ± 0.5	3.0 ± 0.7	2.8 ± 0.4	4.0 ± 0.5	2.0 ± 0.8
UFH4a	1.6 ± 0.5	2.7 ± 0.5	3.8 ± 0.5	2.9 ± 0.8	2.4 ± 0.7	2.5 ± 0.5
UFH4b	1.9 ± 0.6	2.6 ± 0.8	4.1 ± 0.6	2.7 ± 0.8	2.9 ± 0.8	2.2 ± 0.5
UFH4c	2.3 ± 0.6	1.9 ± 0.6	3.8 ± 0.9	2.1 ± 0.7	3.1 ± 0.9	1.5 ± 0.5

ameters in relation to ripening was due to the fact that panelists in every testing session examined samples of the same ripening. Scores given by them were comparative among the three samples tested whereas in the case of mechanical variables the data were absolute values. This is an indication that ripening process in all three samples follows the same pattern. The inability to detect differences between samples from the mechanical parameters was attributed to the low sensitivity of the instrument.

Data from sensory parameters showed that the traditional teleme appeared to be harder than the UF-non heat treated (UFN) teleme which in turn was harder than the UF-heat treated (UFH) sample. The traditional and UFN types showed the same cohesiveness which was greater than that of the third type. The same applied for elasticity where in the traditional teleme appeared to be more grainy than the two UF-types which showed the same smoothness. Traditional teleme was also more brittle than the two UF-types and it was less sticky than the UFN which, in turn, was less sticky than the UFH teleme. The differences between sensory parameters could be attributed to the different technology employed for manufacture of the 3 teleme types. As previously mentioned, apart from application of ultrafiltration, the UF-milk concentrate, in one of the two UF-teleme types, was used unheated whereas the other had been heat-treated. Both the two UF-teleme types retained virtually all the whey proteins, whereas in the traditional teleme, the whey proteins are almost entirely lost during manufacture. Hence, the whey proteins are not very important in the textural behavior of traditional teleme. In the UFN teleme the whey proteins are present in their natural conformation whereas in the UFH teleme are partially denatured due to heating.

Nevertheless, the effect of whey proteins on properties of UF-cheeses is uncertain. Lelievre and Lawrence (1988) stated that the effect of whey proteins depended upon the level and composition of these proteins, whether they were denatured and the extent to which aggregation with casein had occurred.

Table 5—Results of the two-way analysis of variance applied for each of the chemical, instrumental and sensory variables; comparisons of means between type of cheese and ripening period using the SNK test (codes and abbreviations are explained in Tables 1 and 2)

		Influencing factors		
	p-value	Type of cheese	p-value	Ripening
Constituents				
Dry matter	0.726	n.s.	0.861	n.s.
Fat	0.001	TRA>UFN=UFH	0.370	n.s.
Total nitrogen	0.205	n.s.	0.697	n.s.
WSN/TN	<0.001	UFN>UFH>TRA	0.008	$t_4>t_2$[a]
NPN/TN	0.262	n.s.	<0.001	$t_4>t_2$
NCN/TN	0.009	UFN=UFH>TRA	0.003	$t_4>t_2$
Salt	0.240	n.s.	0.540	n.s.
pH	0.015	UFN=UFH>TRA	0.730	n.s.
Mechanical variables				
Hardness	0.072	n.s.	0.045	$t_2>t_4$
Cohesiveness	0.303	n.s.	0.158	n.s.
Springiness	0.311	n.s.	0.560	n.s.
Gumminess	0.051	n.s.	0.024	$t_2>t_4$
Chewiness	0.134	n.s.	0.134	n.s.
Fracturability	0.285	n.s.	0.193	n.s.
Sensory variables				
Hardness	0.001	TRA>UFN>UFH	0.587	n.s.
Cohesiveness	0.005	TRA=UFN>UFH	0.528	n.s.
Springiness	0.002	TRA=UFN>UFH	0.393	n.s.
Adhesiveness	<0.001	UFH>UFN>TRA	0.449	n.s.
Lumpiness	<0.001	TRA>UFN=UFH	0.289	n.s.
Brittleness	0.001	TRA>UFN=UFH	0.564	n.s.

[a] t_2= two months period. t_4= four months period.

The difference in structure of UF-cheeses has been attributed to the fact that undenatured whey proteins do not participate in formation of the casein matrix and may disturb the matrix. They probably simply act as an inert filler in the matrix (de Koning et al., 1981). However, the denatured whey proteins complex with casein micelles which may reduce casein-casein interac-

Table 6—Correlation matrix[a] between mechanical, sensory and chemical variables. Correlation values greater than 0.497 are significant at 0.05 probability level

	HARD M	COHE M	SPRI M	GUMM M	CHEW M	FRAC M	HARD S	COHE S	SPRI S	ADHE S	LUMP S	BRIT S	DM	FAT	SALT	TN	WSN/ TN	NPN/ TN	NCN/ TN
COHE M	0.321																		
SPRI M	0.175	0.214																	
GUMM M	0.909	0.654	0.296																
CHEW M	0.759	0.416	0.541	0.787															
FRAC M	0.748	0.161	0.554	0.646	0.809														
HARD S	0.636	0.511	0.508	0.682	0.659	0.630													
COHE S	0.432	0.670	0.516	0.633	0.574	0.465	0.785												
SPRI S	0.454	0.271	0.392	0.465	0.540	0.460	0.722	0.636											
ADHE S	−0.558	−0.485	−0.388	−0.612	−0.653	−0.513	−0.885	−0.796	−0.856										
LUMP S	0.396	0.253	0.099	0.376	0.210	0.102	0.685	0.374	0.645	−0.669									
BRIT S	0.497	0.281	0.382	0.477	0.474	0.387	0.767	0.492	0.840	−0.826	0.856								
DM	0.133	0.196	0.441	0.281	0.254	0.012	0.198	0.293	0.498	−0.287	0.392	0.539							
FAT	0.477	0.442	0.179	0.552	0.345	0.143	0.657	0.434	0.548	−0.650	0.876	0.806	0.549						
SALT	−0.356	−0.303	−0.111	−0.311	−0.361	−0.519	−0.487	−0.113	−0.050	0.303	−0.177	−0.176	0.486	−0.150					
TN	0.283	0.333	0.284	0.398	0.401	0.063	0.366	0.254	0.621	−0.479	0.632	0.734	0.855	0.740	0.160				
WSN/TN	−0.520	−0.213	0.005	−0.417	−0.348	−0.329	−0.538	−0.102	−0.089	0.331	−0.593	−0.450	0.128	−0.576	0.605	−0.222			
NPN/TN	−0.461	−0.224	−0.270	−0.474	−0.335	−0.413	−0.220	−0.175	−0.040	0.188	−0.134	−0.295	−0.272	−0.380	0.118	−0.278	0.384		
NCN/TN	−0.525	−0.432	−0.237	−0.575	−0.496	−0.331	−0.416	−0.164	−0.292	0.387	−0.531	−0.611	−0.418	−0.729	0.287	−0.676	0.638	0.719	
pH	−0.343	−0.201	0.116	−0.257	−0.146	−0.193	−0.581	−0.196	−0.078	0.378	−0.512	−0.274	0.478	−0.395	0.695	0.164	0.757	−0.089	0.162

[a] Abbreviations are as follows: Hard= Hardness, Cohe= Cohesiveness, Spri= Springiness, Chew= Chewiness, Frac= Fraturability, Adhe= Adhesiveness, Lump= Lumpiness. Brit= Brittleness; M denotes the mechanical (instrumental) variables and S denotes the sensory variables.

tions in the product thereby altering the basic framework (Lelievre and Lawrence, 1988).

Since the strength and firmness of a traditional cheese is due, to a large extent, to the casein framework, inclusion of whey proteins would be expected to affect texture. Thus, UF-Mozzarella was reported to have a softer texture than the traditional cheese (Lelievre and Lawrence, 1988) and UF-Gouda was also significantly softer than the traditional one (Spangler et al., 1990). Similarly, in our work, the UF-types of teleme, appeared to have softer texture than the traditional cheese (Tables 3 and 4). Moreover, cheese from UF milk concentrate, was believed to have a smoother consistency than that from whole milk. The degree of smoothness presumably depended upon the proportion of whey proteins incorporated (Lawrence et al., 1987). The UF-teleme cheeses also appeared to have a smoother texture than traditional teleme (Table 5). This was possibly not only due to presence of whey proteins but also due to the higher pH of the UF types. According to Creamer and Olson (1982) the higher pH cheeses have a texture of a concentrated protein emulsion whereas the lower pH cheeses form porous masses of casein and fat particles. Thereby they acquire a rather grainy texture.

An effort was made to investigate possible correlations among the various parameters. Thus, a correlation matrix was formed which included the instrumental, chemical and sensory variables of the samples regardless of teleme type or ripening time (Table 6). As can be seen, fairly strong correlations in the majority ($r \geq 0.700$—0.850) were obtained only among instrumental variables and among sensory variables themselves, but not between the two groups. Especially, sensory springiness and brittleness unexpectedly showed fairly strong correlation ($r = 0.840$). This could be attributed to the fact that springiness is a primary textural attribute whereas brittleness is a secondary one which derives from the interactions of primary attributes such as springiness, hardness etc. It is likely that for products of very similar type it is very difficult for panelists to distinguish small differences between two attributes. On the other hand, between instrumental and sensory parameters weak positive correlations were obtained between sensory hardness and instrumental hardness, and between sensory cohesiveness with instrumental cohesiveness and gumminess. Adhesiveness negatively correlated with chewiness. Whereas the sensory attributes could differentiate fairly well the three types of teleme cheese, the instrumental parameters could not. This implies that the Instron might not be sensitive enough to trace differences between cheeses of fairly similar type.

Other workers have reported relatively low correlations between sensory and instrumental variables. Thus, Spangler et al. (1990) reported that for UF-Gouda the correlation between Instron hardness and sensory firmness was 0.55, lower than that found in our work (0.636). Jack et al. (1993) reported that no correlations for cheddar cheeses were found between Instron data and sensory parameters and Green et al. (1985) also reported low correlations for the same cheese. However, Chen et al. (1979) found good correlations for sensory and instrumental hardness, cohesiveness, chewiness and adhesiveness for several varieties of cheese and similar results were reported by Lee et al. (1978). Regarding chemical parameters the fat content correlated fairly well with lumpiness and brittleness and the total nitrogen content with brittleness (Table 6).

Redundancy ordination analysis which included instrumental and sensory variables as the response ones and the chemical variables as the cause ones showed how these parameters interacted with cheese samples (Fig. 1). The first two eigenvalues (axes 1 and 2) explained 86.6% of the total variation between the profiles. The length of the arrows indicates the magnitude of the variable effect and the angle between arrows indicates the strength of the correlation between variables (0° and 180° equals to ±1.00 and 90° to zero correlation). Samples close to an arrow indicate particular effect of the corresponding variable.

Textural behavior of each type of teleme cheese was characterized by different attributes. Thus, either sensory springiness or brittleness, lumpiness and fat content characterize the majority of the traditional teleme samples, whereas adhesiveness, pH, water soluble and noncaseinic nitrogen characterize the UFH teleme samples. Moreover, the two cheese types were located on the plane in entirely opposite directions. The behaviour of the UFN teleme is inconclusive, since the various samples show different behaviour and were not clustered. The redundancy analysis confirmed results deduced from analysis of variance. Mechanical and sensory variables acted independently, that is why they formed unique clustered directions (excluding sensory adhesiveness) which did not interact. Strong marginal effects between the bundle-like groups exist, as indicated by strong positive correlation between sensory and instrumental cohesiveness.

Certain variables between sensory and mechanical directions correlate strongly on the plane meaning that they cause similar effect on the teleme cheese. These were: the instrumental springiness with fracturability and instrumental hardness with gumminess; sensory cohesiveness with hardness, sensory springiness with brittleness and both of these negatively with adhesiveness. Also note that gumminess is a far better term to characterize a semi-solid food like cheese than chewiness, as was suggested by Szczesniak (1966) and this was also reported by Lee et al. (1978).

Results from the redundancy analysis were related to those obtained from the 2 way-ANOVA and the correlation matrix.

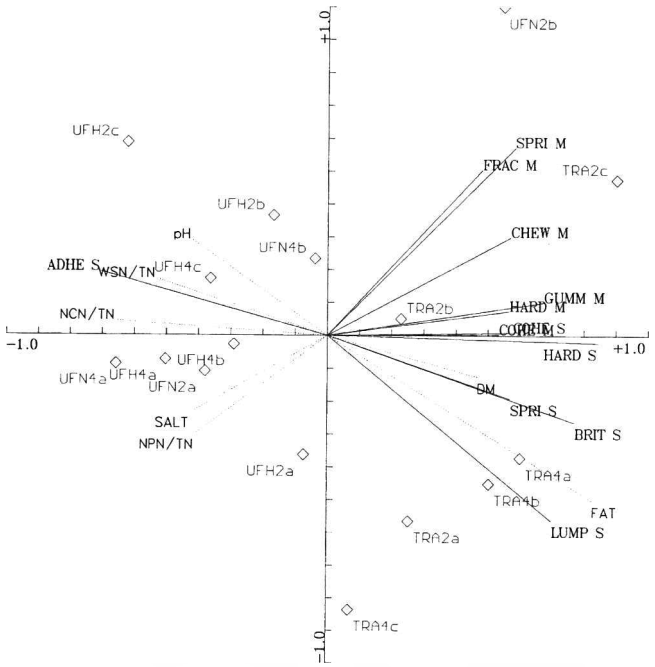

Fig. 1—Redundancy ordination analysis showing cheese distribution as influenced by mechanical, sensory and chemical profiles. Codes and abbreviations are explained in Table 1 and 2.

We could attribute the differences in sensory variables brittleness and lumpiness between the three teleme types (TRA > UFN = UFH) to the higher fat content of the traditional teleme in comparison to UF-cheeses. This was confirmed by high correlations between fat and lumpiness (0.876) as well as fat and brittleness (0.806) (Table 6). The greater adhesiveness of UF-cheeses in relation to traditional teleme could be attributed to their higher pH and higher nitrogen fractions, WSN and NCN. Regarding reported high correlations by Lee et al. (1978) and Chen et al. (1979), between sensory and instrumental textural parameters, note that they referred to a wide variety of cheeses ranging from very soft to very hard ones. Thus, it was possible for the Instron to detect significant differences between the cheeses. When the textural studies were carried out for very similar types of cheese the correlations were low. This was possibly due to the limitations of the Instron. Additional research is needed for further refinement of techniques for textural studies of cheeses.

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Ms received 10/12/94; revised 3/15/95; accepted 5/16/95.

Texture of Cheddar Cheese as Influenced by Fat Reduction

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ABSTRACT

Cheddar cheeses with five different fat levels (34, 32, 27, 21, and 13 %) were manufactured in a randomized block design experiment and replicated four times. Cheeses were ripened for 4 months at 7°C. Microstructure was studied using Scanning Electron Microscopy (SEM). Texture characteristics (adhesiveness, cohesiveness, hardness, and springiness) were determined by Texture Profile Analysis (TPA) using the Instron Universal Testing Machine and a trained sensory panel. Scanning electron micrographs showed that the open-intricate microstructure of the cheeses was lost with a decrease in fat content. Hardness and springiness increased while adhesiveness and cohesiveness of the cheeses decreased with decreasing fat content. Texture attributes were influenced by the nature of the protein matrix that resulted due to fat removal.

Key Words: texture, cheddar cheese, fat, microstructure

INTRODUCTION

TEXTURE is an important characteristic of Cheddar cheese in determining consumer acceptability and quality (McEwan et al., 1989). Much research has been published on cheese texture. Chen et al. (1979) used the Instron to evaluate 6 textural characteristics (hardness, cohesiveness, adhesiveness, springiness, gumminess and chewiness) of 11 cheese varieties ranging from Parmesan to cream cheese. Measurements closely correlated with composition and pH. Green et al. (1981) analyzed textural characteristics of Cheddar cheese made from concentrated milk using the Instron. Instrumental firmness, cohesiveness, force required for fracture and adhesiveness increased as concentration of milk increased, whereas springiness did not change. Stampanoni and Noble (1991) used the Instron to evaluate the effects of fat, acid and salt on textural attributes of cheese analogs. Analogs containing higher amounts of fat were softer, less springy, and more cohesive and adhesive. Increasing acid or salt increased firmness, but decreased cohesiveness and springiness.

Correlations of instrumental analysis and sensory data, are limited to a particular product or cheese variety, and often cannot be extrapolated to other products. Instrumental measurements have not always correlated well with sensory analysis (Eves et al., 1988; Jack et al., 1993). Some reasons for seeking correlation, difficulties encountered and developments in this area were reviewed by Szczesniak (1987).

Brennan et al. (1975) studied three mechanical properties of five cheeses, using both the Texturometer and Instron, and reported the highest correlation coefficient was between sensory evaluation and the Instron. Stampanoni and Noble (1991) observed a positive correlation between sensory and instrumental measurements in studies with cheese analogs. Lakhani et al. (1991) found no correlation between instrumental and sensory characteristics of cheddar cheese made from ultrafiltered milk.

Poor correlation of instrumental measurements with sensory data may be partially due to uncontrolled surface friction between sample and compression plates. Culioli and Sherman (1976) reported when firmness was evaluated, results depended on the compression behavior of the samples as modified by frictional effects. Goh and Sherman (1987) investigated stress relaxation behavior of Gouda cheese using unlubricated and lubricated plates. Other studies include the effect of lubrication of samples with mineral oil (Ak and Gunasekaran, 1992) or bonding of samples with cyanoacrylate ester adhesive (Casiraghi et al., 1985). Brennan and Bourne (1994) reported on cheese and frankfurter samples that were subjected to a 50% uniaxial compression between flat plates and artificial molars, mounted on a Texture Analyzer. They concluded that the frictional effect of the cusps on the molar teeth was greater than the effect of lubrication. Observations made when samples were compressed between the molar teeth confirmed their observation that compression of foods in the mouth followed the nonlubricated pattern although lubrication was provided by saliva.

Cheese texture is largely determined by microstructure, which on natural cheeses has been studied extensively using Transmission Electron Microscopy and Scanning Electron Microscopy. Emmons et al. (1980) observed the microstructure of full and reduced fat cheeses made from homogenized milk. Green et al. (1981) observed microstructural changes in cheddar cheese made from concentrated milk. Kiely et al. (1993) studied age related changes in microstructure of mozzarella cheese and reported that porosity of the paracasein matrix decreased through ripening. They attributed growth of matrix cavities to proteolytic destruction of protein. Taneya et al. (1992) observed the microstructure of string cheese concluding that stringiness was significantly associated with a uniform, longitudinal orientation of the protein matrix, and fat was dispersed between the protein strands.

Thirty-six percent of all reduced fat dairy products introduced in 1992 were cheeses (O'Donnell, 1993). Poor texture, slow flavor development and poor keeping quality have been common problems in reduced fat cheeses. Milk fat makes multiple contributions to cheese texture, but not all are well understood. Reduced-fat cheeses tend to be harder, more elastic and more adhesive than their full fat counterparts (Schulz et al., 1952; Kay, 1965; Davis, 1965; Emmons et al., 1980; Lawrence and Gilles, 1987; Olson and Johnson, 1990). Increasing moisture content has been generally recommended to improve texture of reduced fat cheeses. Our objective was to investigate the influence of reduction in milk fat on microstructure and on perception of texture attributes in cheddar cheese, and to correlate sensory data with measurements from the Instron Universal Testing Machine.

MATERIALS & METHODS

Cheese manufacture

Cheddar cheese with varying fat levels, 34, 32, 27, 21, and 13%, was manufactured from standardized milk with 3.8, 3.2, 2.4, 1.6, and 0.8% fat respectively. Milk was warmed to 31°C and inoculated with 0.02% DVS (Direct Vat Set) culture (DVS #980, Chr. Hansen's Laboratory, Inc., Milwaukee, WI). Milk was ripened for 1 hr, and 0.01% chymosin (Chymax-Double strength, Pfizer, Milwaukee, WI) was added to clot milk in 30 min. Cheddar cheese was manufactured by the standard procedure outlined in Kosikowski (1982). Curd for all cheeses was milled at the same pH. It was salted, hooped and pressed for 18 hr. Manufactured cheeses were vacuum packaged the next day and ripened at 7°C for 4 mo. Cheese manufacturing for all treatments was replicated four

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times. Cheeses were assayed for fat using the Babcock procedure, protein using the Kjeldahl method and moisture, as described in Standard Methods for Examination of Dairy Products (Marshall, 1992).

Instrumental texture profile analysis

Cheddar cheese was cut into cylindrical samples of 20 × 20 mm using a cork borer and a wire cutter, placed in plastic cups, sealed (to prevent dehydration), and stored at 9°C overnight. A two-bite compression test was performed using the Instron Universal Testing Machine (Model 4202, Canton, MA) with a 10 kN load cell. An 80% compression was used and crosshead speed was 10 mm/min. Samples were lubricated by placing one drop of vegetable oil on the top and bottom surfaces. Hardness, cohesiveness, and adhesiveness were determined in triplicate from the texture profile curve as described by Bourne (1967). Springiness was determined in triplicate using a 55% compression test similar to that described by Stampanoni and Noble (1991). Height of the sample was measured before and after compression. Springiness was expressed as percent of sample returned to its original height.

Sensory determination

Texture was evaluated using a 15-member trained sensory panel consisting of faculty and graduate students at Michigan State University. They were selected through a screening process for ability and reliability to distinguish the tested cheese attributes. The panelists participated in one orientation and four training sessions. They were trained to judge cheese texture as described by Civille and Szczesniak (1973). The training involved practice with 20 different cheeses purchased at local grocery stores that varied in sensory attributes being tested. Panelists also practiced using the structured rating scale to quantify tested attributes and were provided feedback on their ratings. Data collection sessions were held twice a day with five test samples evaluated/session. A reference sample was also provided at each session. All training and data collection was done in a climate-controlled sensory analysis laboratory equipped with individual testing booths. Panelists were provided water (at room temperature) and unsalted crackers for rinsing and palate cleaning. Cheese samples were cut into 20 × 20 mm cylinders the day before testing and stored in covered plastic cups at 9°C overnight. The cups were identified by random 3-digit numbers.

The panel was instructed to evaluate samples for adhesiveness, cohesiveness, hardness and springiness. To determine adhesiveness, panelists were advised to place the sample between the molars, chew it five times, press it with the tongue to the roof of the mouth and evaluate the force required to remove sample with the tongue. Cohesiveness was evaluated by placing the sample between molars, compressing it fully and noting the degree to which it deformed rather than crumbled or fell apart. To determine hardness panelists were advised to place the sample between molars, bite through it once and evaluate the force required to bite into the cheese. For springiness, the panelists were advised to place the sample between molars, compress it partially without breaking the structure, release the tooth pressure and evaluate the degree to which the cheese returned to its original shape. Panelists evaluated each characteristic using a structured 9-point intensity scale, where 1 indicated the lowest and 9 the highest intensity of an attribute. Each attribute was rated on a separate ballot. Sensory texture scores were averaged for 15 judges for each treatment (for all 4 replicates) and attribute tested.

Microstructure determination

Microstructure was evaluated using Scanning Electron Microscopy (SEM). Cheese slices were cut and primary fixed in 4% buffered gluteraldehyde for 1.5 hr, washed in 0.1M phosphate buffer for 30 min. They were post fixed in buffered osmium tetroxide for 2 hr and washed for 30 min in 0.2M phosphate buffer. Samples were dehydrated in increasing concentrations of ethanol and water solutions, 25%, 50%, 75%, and 100% for 30 min in each solution. They were then quick frozen and fractured in liquid nitrogen to expose an uncut surface and placed in 100% ethanol. Samples were critical point dried in a Balzers Critical Point Dryer and coated with a thin layer of gold in an Emscope Sputter Coater EM 500. A JEOL Scanning Electron Microscope, at 15 KV accelerating voltage, was used to view each sample. Microstructure of all the cheeses was evaluated. Micrographs of reprensentative replicates were selected for presentation.

Statistical analysis

The experiment was replicated four times in a randomized block design. Days were blocked. All tests for TPA were conducted in triplicate. Sensory Analysis was done using 15 trained panelists. Microcomputer Statistical Program (MSTAT) (Michigan State University, E. Lansing, MI) was used for statistical analysis of the data, to determine differences between treatment means and correlation coefficients. A separate ANOVA was conducted for each characteristic tested. Treatment means were considered significantly different at $P \leq 0.05$ unless differently stated.

RESULTS & DISCUSSION

COMPOSITIONAL DATA of the manufactured cheeses showed, as expected, that moisture and protein content of the cheeses increased ($P < 0.05$) when fat was removed. Moisture content (wet basis) of the cheeses containing 34 ± 2.9, 32 ± 2.3, 27 ± 1.4, 21 ± 1.9, and 13 ± 1.9% fat were 38.5 ± 1.34, 39.7 ± 1.89, 40.8 ± 1.35, 40.8 ± 1.81, and 44.7 ± 2.16%, respectively. Protein contents of these cheeses were 22.3 ± 1.74, 24.3 ± 1.06, 27.9 ± 1.53, 32.7 ± 1.26, and 36.4 ± 1.06%, respectively. Cheddar cheese is a viscoelastic solid in which the caseins form a protein network with entrapped fat and moisture. The level of milk fat in the cheese had a significant effect on the protein matrix as determined by SEM (Fig.1), thus, impacting various textural attributes (Tables 1 and 2).

Hardness. Hardness (force necessary to attain a given deformation) is most commonly evaluated when determining cheese texture. Hardness of cheddar cheese, as determined both by Instron and sensory panel increased with decrease in fat content (Table 1 and 2). However, cheeses containing 34, 32, and 27% were similar in hardness as determined by Instron. When the fat level in the cheese was reduced to 21%, hardness increased ($P < 0.05$). The cheese containing 13% fat was the hardest ($P < 0.05$) (Table 1).

Cheeses containing 34 and 32% fat received similar hardness scores by the trained sensory panel. Judges scored the cheeses as increasing in hardness ($P < 0.05$) as fat level decreased (Table 2). There was a positive, and significant correlation ($r = 0.95$), between Instron determination of hardness and sensory ratings ($P < 0.01$).

Scanning electron micrographs indicated that cheeses with 34 and 32% fat had similar microstructures. The protein matrix was loose and open with spaces occupied by the fat globules dispersed through the protein network providing a lace-like appearance (Fig. 1). Microstructure of the cheese containing 27% fat was distinctly different from cheeses with 34 and 32% fat. This cheese had a more compact protein matrix with less open spaces that would be occupied by milk fat globules. Compact appearance of the protein network increased and number of milk fat globules dispersed within the network decreased with reduction in fat content of cheeses. This probably explained the hard texture observed with the lower fat cheeses (Tables 1 and 2), even though they were significantly higher in moisture content. Banks et al. (1989) observed an increase in hardness of reduced fat Cheddar cheese containing 25% and 16% fat and 42.9% and 47.2% moisture, respectively, compared to their full fat counterparts. Stampanoni and Noble (1991) also observed an increase in hardness of cheese analogs (manufactured with rennet casein, deionized water and melted vegetable fat) with decrease in fat content. Prentice (1987) reported a direct relationship between firmness and cheese moisture. Tunick et al. (1991) reported on increase in the hardness of Mozzarella cheese with decreased fat content. The effect was more pronounced at higher cooking temperatures. Their study also showed that reduced moisture levels resulted in greater hardness. They hypothesized that firmer texture was due to the alteration in the casein matrix, as well as water acting as either a lubricant or plasticizer between the proteins. Our micrographs showed that higher moisture cheddar cheeses (with reduced fat) had a compact very dense protein matrix. Higher moisture content of these cheeses

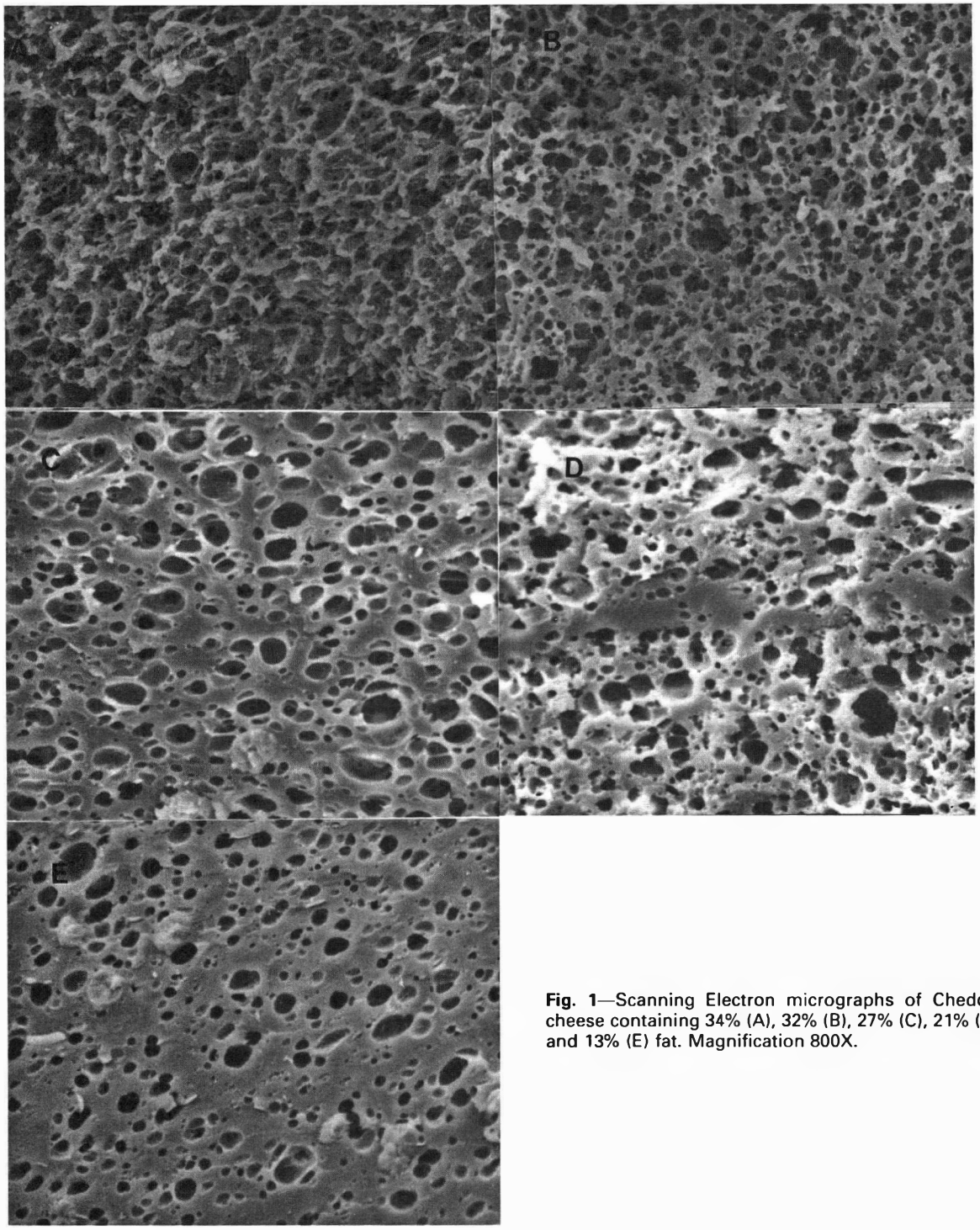

Fig. 1—Scanning Electron micrographs of Cheddar cheese containing 34% (A), 32% (B), 27% (C), 21% (D), and 13% (E) fat. Magnification 800X.

did not soften the protein matrix. The removal of fat altered the protein matrix resulting in a compact and dense appearance. Chen et al. (1979) showed that hardness of cheese varieties with varying composition correlated most closely with protein content and not fat or moisture. The ranges they tested were: fat 18–32% and moisture 44–30%.

Springiness. Springiness is the rate and extent to which a deformed material goes back to its undeformed condition after the deforming force is removed (Civille and Szczesniak, 1973). Springiness of the cheeses increased with decrease in fat content as determined by both the Instron and sensory panel. Cheddar cheeses containing 34 and 32% fat were similar in springiness as determined by Instron. When the fat level in the cheese was reduced to 27, 21 and 13 % it became springier ($P < 0.05$). The trained sensory panel also could not detect differences in springiness between cheeses containing 34 and 32% fat. The sensory panel scored cheese as increasing in springiness ($P < 0.05$) for cheese containing 27%, 21%, and 13% fat. The Instron determination of springiness positively and highly correlated ($r = 0.95$), with sensory determination of springiness ($P < 0.01$). The protein matrix was more flexible and elastic as fat was removed shown by springiness scores (Tables 1 and 2) regardless of higher moisture of the reduced fat cheeses. Stampanoni and Noble (1991) observed an increase in springiness of cheese analogs with decrease in fat. Tunick et al. (1993) also reported an increase in springiness of mozzarella cheese with decrease in fat. A linear decrease in elasticity of mozzarella cheese was also reported as the ratio of fat to solids-not-fat increased (Masi and Addeo, 1986). Emmons et al. (1980) demonstrated low fat cheddar cheese was springier than full fat cheddar cheese hypothesizing that reducing fat content resulted in fewer fat globules with more casein being deformed per unit volume evidenced by

Table 1—Influence of milk fat on texture profile analysis (TPA) parameters of Cheddar cheese as determined by Instron

Treatments Cheese fat (%)	Adhesiveness (N mm)	Cohesiveness (ratio)	Hardness (N)	Springiness (%)
34	1.15[a] (0.31)	0.14[a] (0.03)	193.7[a] (66.5)	58.1[a] (3.3)
32	1.13[a] (0.40)	0.13[a] (0.03)	260.7[a] (95.4)	57.8[a] (7.2)
27	1.18[a] (0.36)	0.15[a] (0.02)	280.0[a] (60.3)	71.5[b] (6.0)
21	0.53[b] (0.44)	0.19[b] (0.03)	468.7[b] (98.9)	78.9[c] (8.2)
13	0.49[b] (0.40)	0.22[b] (0.04)	762.3[c] (96.5)	88.1[d] (6.5)

[a-d] Means with standard deviations in parentheses. Means with the same superscript are not significantly different ($P < 0.05$). Comparisons are made only within the same column. Treatments were replicated four times, each test was conducted in triplicate.

Table 2—Influence of milk fat on texture characteristics of Cheddar cheese as determined by a trained sensory panel

Treatments Cheese fat (%)	Adhesiveness	Cohesiveness	Hardness	Springiness
34	6.9[a] (1.6)	5.7[a] (2.6)	2.7[a] (1.2)	2.6[a] (1.5)
32	6.3[a] (1.7)	5.0[abc] (2.2)	2.9[a] (1.6)	2.5[a] (1.5)
27	5.3[b] (1.9)	5.3[ab] (2.0)	4.1[b] (1.7)	3.6[b] (1.8)
21	4.0[c] (2.1)	4.7[abc] (1.6)	5.6[c] (1.5)	5.3[c] (1.9)
13	2.1[d] (1.3)	4.5[bc] (2.1)	7.0[d] (1.4)	6.9[d] (1.7)

[a-d] Means with the same superscript are not significantly different ($P < 0.05$). Comparisons are made only within the same column. Means with standard deviations in parentheses; n = 60 for all treatments (4 replicates × 15 judges).

electron micrographs. However, increasing homogenization pressure decreased elasticity of reduced fat cheeses due to the higher moisture content (Emmons et al., 1980). Tunick et al. (1991) reported greater values for springiness with reduced moisture levels in mozzarella cheese. Our results showed that increase in moisture did not decrease springiness of reduced fat cheddar cheese. This was consistent with the observation of Chen et al. (1979), that protein level was the dominant component affecting elasticity of cheese varieties.

Adhesiveness. Adhesiveness (work necessary to overcome attractive forces between surface of cheese and surface of contacting material) of cheeses decreased with decreasing fat content (Table 1). However, adhesiveness values for cheeses containing 34, 32, and 27% fat were similar. These scores were higher ($P < 0.05$) than for cheeses containing 21 and 13% fat. The sensory panel evaluated the force required to remove the cheese that adhered to the roof of the mouth (Civille and Szczesniak, 1973). Adhesiveness scores decreased as fat content decreased (Table 2). Cheeses containing 34 and 32% fat were similar in adhesiveness and were the most adhesive ($P < 0.05$) among those tested. Cheeses containing 27, 21, and 13% fat decreased ($P < 0.05$) in adhesiveness with decreasing fat content. The Instron determination of adhesiveness also correlated positively ($r = 0.73$) with sensory determinations ($P < 0.01$). For high correlation between instrumental and sensory analysis many factors are critical: uniform lubrication of compression plates, uniform sample size and geometry, % deformation, and surface friction.

Scanning electron micrographs indicated that the most adhesive cheeses were those containing an open and loose protein matrix as in the higher fat cheeses (Fig. 1). As the protein matrix became increasingly more compact the cheeses lost adhesiveness (Fig. 1; Tables 1 and 2). Olson and Johnson (1990) reported low-fat cheeses exhibiting a higher degree of stickiness when masticated. This was particularly evident in samples with fat contents of 15% or less and after aging. Since moisture contents of low-fat cheeses are higher and proteins are degraded during ripening such cheese were expected to be more adhesive. However, in our study low fat cheeses that were ripened for 4 mo were less adhesive regardless of moisture content. According to our micrographs, removal of fat which increased protein content, altered the protein matrix, making it more compact and therefore less adhesive. Protein content has been the dominant factor influencing adhesiveness of cheese with varying composition (Chen et al., 1979).

Cohesiveness. As fat content of cheeses decreased, cohesiveness (the extent to which they could be deformed before they ruptured) increased as determined by Instron (Table 1). Cheeses containing 34, 32, and 27% fat were similar in cohesiveness. Cheeses containing 21 and 13% fat were also similar in cohesiveness and were more cohesive than those containing 34, 32, and 27% fat. In contrast, sensory cohesiveness decreased as fat content decreased (Table 2), but not consistently. Only the 34 and 13% fat cheeses differed in cohesiveness. Stampanoni and Noble (1991) reported a decrease in cohesiveness with decreased fat content of cheese analogs. Tunick et al. (1991) reported that reduced moisture content in mozzarella cheese decreased its cohesiveness.

The nature of the protein matrix and the extent of fat dispersion may contribute to cohesiveness or the tendency of cheese to adhere to itself (Fig. 1, Tables 1 and 2). Cohesiveness is somewhat difficult to evaluate, because as fat was removed the cheese became more springy. A springy cheese resisted deformation and did not rupture easily, thus it was more cohesive by Instron measurement. However, the more compact reduced fat cheeses seemed to crumble and break more easily according to sensory panelists, thus they were perceived as less cohesive. It is also possible that the very slow speed of the Instron and the flat compression plate compared to the sharpness of molars and speed of the teeth in the sensory evaluation was responsible for the low correlation between the two in this respect. Instrumental and sensory determinations for cohesiveness did not correlate ($r = -0.41$).

CONCLUSION

AS FAT IN CHEESE DECREASED, and moisture increased, hardness and springiness increased and adhesiveness and cohesiveness decreased. This occured although moisture increased. Scanning electron micrographs indicated that the nature of the protein matrix, which is affected by fat content of the cheeses, influenced texture attributes. Manufacturing acceptable, reduced fat cheeses requires controlling the dense microstructure that results from high concentration of protein undisrupted by fat. Disruption of the casein-casein interactions by fat to loosen the protein matrix appears essential to achieve desirable texture. Increasing moisture content or modification of milk fat globules alone did not improve cheddar cheese texture.

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Oxytetracycline, Sulfadimethoxine, and Ormetoprim Residues in Channel Catfish by HPLC

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ABSTRACT

HPLC methods and modified extraction procedures were used to analyze residues of oxytetracycline (Terramycin, OTC) or sulfadimethoxine (SDM) and ormetoprim (OMP) in channel catfish fed OTC or Romet-30 under controlled conditions. Mean recovery rates in fish muscle were 92.5% for OTC over concentrations of 0.05–1.0 ppm and 86.3% for OMP and 87.9% for SDM over 0.05–5.0 ppm. SDM and OMP were rapidly depleted from large catfish (about 345g) after 5 days feeding with Romet-30 at 50–100 mg/kg body weight. By day 2 post-treatment, no residue was detected. Residues of OTC, SDM and OMP were also detected in fingerling channel catfish (about 20.3g) after 4 and 8 wk of feeding with Romet-30 or OTC at 12.5, 25, or 50 mg/kg body weight, but not after a 3-wk-withdrawal period.

Key Words: channel catfish, oxytetracycline, sulfadimethoxine, ormetoprim, HPLC

INTRODUCTION

TERRAMYCIN (oxytetracycline) and Romet-30 (sulfadimethoxine and ormetoprim in a 5:1 ratio) are antibiotics approved by the Food and Drug Administration (FDA) for treating bacterial or coccidial infections in channel catfish. Oxytetracycline (OTC) is a broad-spectrum antibiotic routinely used in catfish aquaculture to control bacterial hemorrhagic septicemia caused by *Aeromonas liquifaciens* and *A. hydrophila* and pseudomonas disease by *Pseudomonas liquifaciens* (Long et al., 1990). It is usually administered at 50 mg/kg fish/day for 10 consecutive days in medicated feed. Sulfadimethoxine (SDM) is a relatively potent and long-acting sulfonamide that is effective when used alone or as a 5:1 combination with ormetoprim (OMP) in treating catfish enteric septicemia caused by *Edwardsiella ictaluri* (Tucker and Robinson, 1990). The drug is typically administered in medicated feed at 50 mg/kg fish/day for 5 consecutive days.

Treatment with antibiotics may result in residues if a proper withdrawal time has not been observed for treated fish. The presence of antimicrobial agents in muscle foods may pose a potential health threat to consumers, especially hypersensitive individuals who may experience allergic reactions to specific antibiotics. Thus, federal law has established a maximum legal tolerance level of 0.1 ppm (mg/kg) for OTC in catfish tissue. It also has established a 21-day withdrawal period following OTC treatment and a 3-day withdrawal time for SDM and OMP (Ruth and Reed, 1991).

No approved official methods are available for determination of OTC, SDM or OMP residue in catfish. Most classic assay methods for antibiotics are time consuming. They lack specificity and require each drug be assayed individually. High-performance liquid chromatography (HPLC) provides selectivity and increased sensitivity over many other analytical procedures. It has been widely used to determine antibiotics in fish, meat, milk and poultry. Two HPLC methods have been reported for determination of OMP and SDM in catfish and salmon tissues (Weiss et al., 1987; Walisser et al., 1990), one for analysis of SDM and OMP in animal feeds (Duke et al., 1993), and several others for OTC analysis in rainbow trout, salmon, and shellfish (Carignan et al., 1993; Murray et al., 1987; Nordlander et al., 1987; Pouliquen et al., 1992; Reimer and Young, 1990). However, no HPLC method has been published for analysis of OTC residues in catfish.

Michel et al. (1990) and Plakas et al. (1990) studied the kinetics of absorption, distribution, metabolism and excretion of SDM and OMP in catfish. Plakas et al. (1988) also studied the disposition and bioavailability of tetracycline in catfish. However, those studies did not reproduce aquacultural feeding conditions during evaluation of the drugs. SDM and OMP were dosed to anesthetized fish either intravascularly via the caudal vein, orally in gelatin capsules, or using a tube-and-plunger apparatus (Michel et al., 1990; Plakas et al., 1990). Milner et al. (1994) demonstrated that levels of drug residue in fillets declined rapidly after catfish were treated with Romet-30 at the recommended level of 50 mg/kg for 5 days. However, information on OTC metabolism and residue levels in catfish under natural feeding conditions as in an aquaculture operation is not available. Therefore, our objectives were to develop specific HPLC procedures for determining OTC, SDM and OMP in catfish tissues and apply these procedures to fish that had been exposed to various concentrations of OTC and Romet-30 under controlled conditions.

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MATERIALS & METHODS

Fish samples

Channel catfish fillets used as blanks and for fortification to determine recovery rate were purchased from a local seafood store. Channel catfish treated with OTC or Romet-30 were provided by Dr. Delbert Gatlin of the Dept. of Wildlife & Fisheries Sciences, Texas A&M Univ. (College Station, TX). Additional channel catfish treated with Romet-30 were also provided by Dr. Wilmer A. Rodgers, Dept. of Fisheries & Allied Aquacultures, Auburn Univ. Fish fillets were frozen and shipped on dry ice to the Food Science & Human Nutrition Dept., Univ. of Florida for analysis.

Catfish treated with OTC or Romet-30 at Texas A&M Univ.

Different levels of Romet-30 and OTC were fed to juvenile channel catfish at the Aquacultural Research & Teaching Facility, Dept. of Wildlife & Fisheries Sciences, Texas A&M Univ. The basal diet (0% antibiotic) was formulated from purified ingredients (25.9% casein, 6% gelatin, 31.6% dextrin, 18% cellulose, 5% menhaden fish oil, 5% catfish offal oil, 4% mineral premix, 3% vitamin premix, 1% $CaHPO_4 \cdot H_2O$, and 0.5% carboxymethylcellulose) to contain 30% crude protein and 3.44 kcal available energy/g. The 1:1 mixture of menhaden fish oil and channel catfish offal oil enhanced the palatability of medicated diets. This diet formulation adequately met all known nutritional requirements of channel catfish (National Research Council, 1993). Six medicated diets containing three concentrations of Romet-30 or OTC to provide 12.5, 25, or 50 mg drug/kg body weight were prepared from the basal diet by substituting the cellulose in the basal diet with these antibacterial agents.

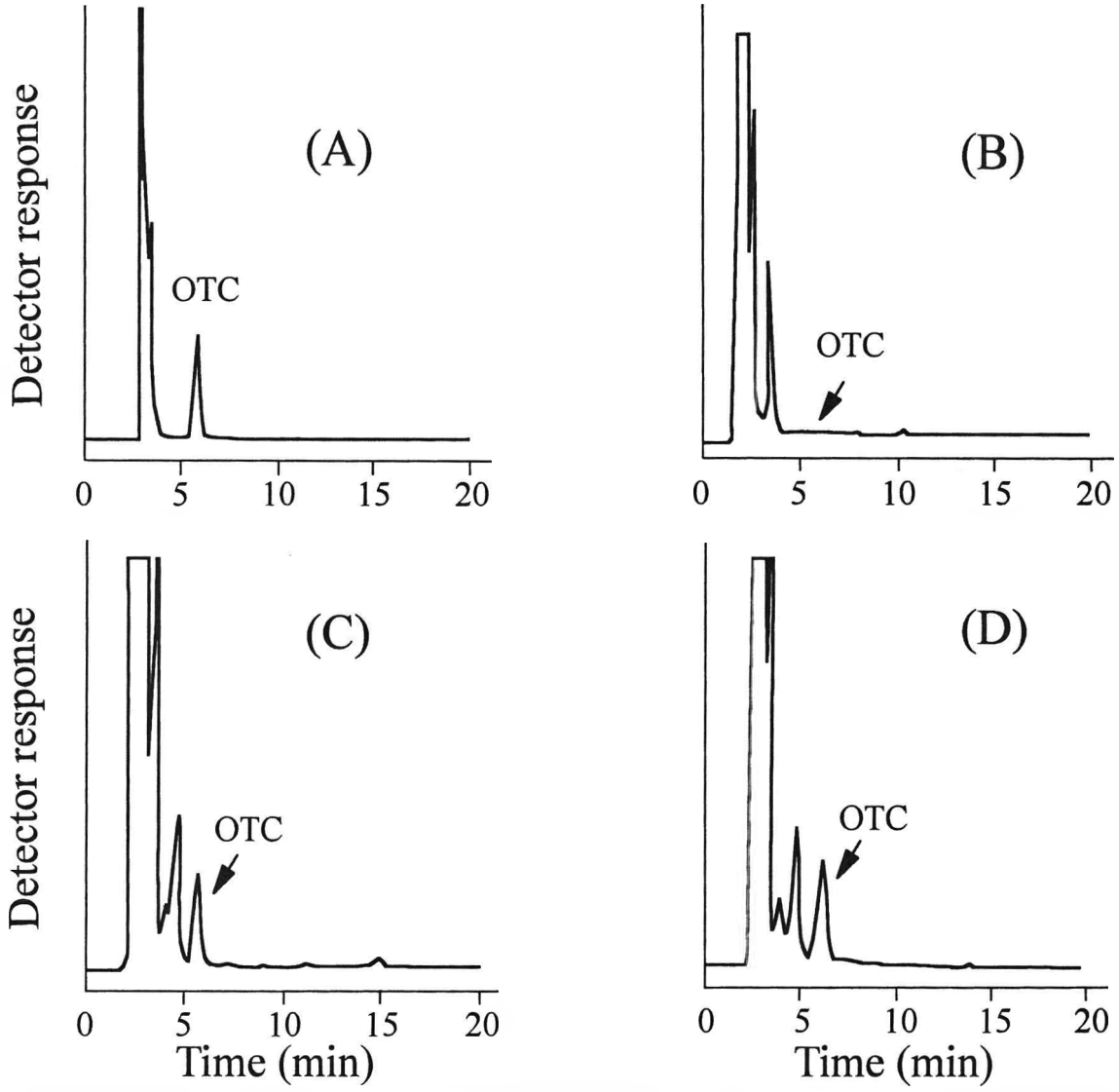

Fig. 1—Typical chromatograms of (A) 1.0 ppm OTC standard solution, and muscle extracts of (B) an untreated catfish, (C) an untreated catfish augmented with 0.2 μg OTC/g, and (D) an OTC-treated catfish. HPLC conditions: see Materials & Methods.

Table 1—Recovery of oxytetracycline from fortified catfish muscle

Fortification level (ppm)	Conc. found (ppm × 10^{-1})	Average recovery (% mean ± SD, n=4)	Coefficient of variation (%)
0.05	0.47	93.8 ± 10.5	11.2
0.1	0.99	99.3 ± 4.5	4.5
0.2	1.90	94.9 ± 6.7	7.1
0.5	4.43	88.5 ± 6.3	7.1
1.0	8.61	86.1 ± 2.2	2.6
	Mean	92.5 ± 5.3	5.7

Fingerling channel catfish with initial body weights of 20.2–20.4g were held indoors in 38-L flow-through aquaria and conditioned on the basal diet for 2 wk. The flow-through aquarium system was provided with well water at a constant flow rate of 0.3–0.4 L/min with a temperature of 28 ± 2°C. Supplemental aeration in all aquaria maintained oxygen concentrations at or near saturation. Fluorescent, overhead lighting was timer-controlled to maintain a 12 hr:12 hr diurnal period.

After conditioning, fingerlings were randomly assigned to aquaria with 10 fish per aquarium. Three replicates were used for each treatment group and were randomly assigned to aquaria. Diets were fed on a dry-matter basis at 3% body weight per day. Daily rations were divided into two equal feedings (AM and PM), 7 days a week. Weight gain, feed efficiency, and feed allowance were determined through weekly weighing each group of fish. Experimental diets were fed for 8 wk followed by an additional 3-wk "withdrawal period" during which all treatments were provided the basal diet.

Two fish were sampled from each replicate group on weeks 4, 8, and 11 of the experimental period. The fish were skinned and fillets from each fish were wrapped separately in aluminum foil and packaged in a resealable plastic bag labeled with date and sample code. The samples were immediately frozen at −17°C and shipped frozen to the Food Science and Human Nutrition Dept., Univ. of Florida for residue analyses.

Catfish treatment with Romet-30 at Auburn Univ.

Sixty channel catfish with mean body weight of 345.0 ± 11.6g (mean ± S.D.) were divided into four groups of 15 fish each and placed in four separate 500-L troughs at the Fisheries Experiment Station, Auburn University, AL. The fish were allowed to acclimate in troughs for 15 days.

Water quality features were checked during the experiment. Water temperature was measured with a maximum/minimum thermometer (Fisher Scientific, Pittsburgh, PA). Dissolved oxygen was monitored with a Model 57 oxygen meter (Yellow Spring Instruments, Yellow Spring, OH). The pH was measured with a Model 7 pH meter (Corning Medical, Medfield, MA). Total alkalinity and hardness were determined using a Hach water quality test kit (Hach Chemicals, Loveland, CO).

Commercial floating catfish feed (Alabama Food Mills, Inc., Tuscaloosa, AL) containing 32% crude protein, 4.0% fat, and 7.0% fiber was ground into a powder, and mixed with a 30% Romet-30 premix (Romet-30, Hoffmann-La Roche Inc., Nutley, NJ) to prepare Romet-medicated feed. Each pound of Romet-30 premix contained 113.5g of SDM and 22.7g of OMP. Three medicated feeds containing 2.5, 3.75, and 5.0g Romet-30/kg feed were prepared from the commercial feed which served as the control. The control and medicated feeds were provided to fish at 2% body weight per day for 5 days, half at 9 AM and the other half at

Table 2—Oxytetracycline residues (ppm) in treated catfish at different feeding intervals

Feeding period	Dose (mg/kg)	Fish number 1	2	3	4	5	6	Average
4 wk	Control	ND[d]	ND	ND	ND	ND	ND	ND
	12.5	0.04	0.09	0.09	0.06	0.04	0.05	0.06 ± 0.02[a]
	25.0	0.18	0.14	0.14	0.10	0.08	0.08	0.12 ± 0.04[bc]
	50.0	0.18	0.15	0.20	0.19	0.12	0.12	0.16 ± 0.04[c]
8 wk	Control	ND	ND	ND	ND	ND	ND	ND
	12.5	0.08	0.09	0.04	0.05	0.07	0.04	0.06 ± 0.02[a]
	25.0	0.11	0.1	0.10	0.10	0.13	0.03	0.10 ± 0.03[b]
	50.0	0.18	0.18	0.12	0.16	0.15	0.51	0.22 ± 0.15[c]

[a-c] Mean ± standard deviation followed by the same letter was not significantly different from each other at P = 0.05 using Tukey's test.
[d] Not detected. For the calculations of averages and standard deviations, ND was assumed to be zero.

4 PM. These feeds provided 0, 50, 75 and 100 mg Romet-30/kg of fish each day.

Three fish were randomly sampled from each group at 24, 48, 72, 96, and 120 hr after the 5-day feeding cycle. The fish were skinned and fillets from each fish were wrapped separately in aluminum foil and packaged in a resealable plastic bag labeled with date and sample code. They were immediately frozen at −17°C and sent to the Univ. of Florida for residue analysis.

Chemicals and expendable materials

HPLC grade acetonitrile and methanol were purchased from Baxter (McGaw Park, IL). Water used for HPLC analyses was distilled and further purified using a Photronix Water System (Reagent Grade: Photronix Corp., Medway, MA).

OTC stock solution was prepared by dissolving 27.62 mg OTC hydrochloride (containing 905 µg oxytetracycline/mg; Sigma Chemical Company, St. Louis, MO) in a 25 mL volumetric flask with methanol. Fortification solution at 100 µg/mL was prepared by diluting the stock solution with methanol. Standard OTC solutions (0.25, 0.5, 1.0, 2.5, 5.0, and 10.0 µg/mL) for HPLC analysis were prepared by diluting the fortification solution with methanol:1M HCl (1:1).

OMP and SDM stock solutions were prepared by dissolving 100 mg each of OMP and SDM in a 100 mL volumetric flask with methanol. OMP and SDM were provided by Dr. Alexander MacDonald of Hoffmann-La Roche Inc. (Nutley, NJ). Fortification solutions (10 and 100 µg/mL) for both compounds were prepared by diluting the stock solution with methanol. Solutions (0.1, 0.2, 0.25, 0.5, 1.0, 2.0, 5.0, 10.0, and 25.0 µg/mL) used to derive standard curves were prepared by diluting fortification solutions with methanol.

Extraction of OTC from fish samples

A modified method of Nordlander et al. (1987) using ethylenediaminetetraacetic acid (EDTA, disodium), 50% trichloroacetic acid (TCA), and 1M HCl was developed to enhance OTC extraction from catfish samples. OTC-treated fish samples, augmented samples used to check daily recovery rates, and blanks were extracted similarly. Catfish fillets were chopped into a fine mince with a knife. Each 5-g minced fish sample was placed in a 50-mL centrifuge tube and OTC fortification solution was added so that the final augmented levels were at 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 ppm. After 2 mL of 50% TCA, 30 mL of 1M HCl, and 0.5 g of EDTA were added, the sample was manually mixed for 3 min with a glass rod, and then mixed using a vortex at high speed for 30 sec. Samples were centrifuged at 920 × *g* for 10 min, and the supernatant filtered through a folded Whatman #1 filter paper. The pellet was then re-extracted with 1 mL of 50% TCA and 15 mL of 1M HCl. All filtrates were pooled in a 50-mL glass tube, and passed through Sep-pak C_{18} cartridges (Waters Assoc. Milford, MA) attached to a PrepTorr vacuum box (Fisher Scientific) for OTC adsorption. The C_{18} cartridge was activated by adding 10 mL methanol followed by another washing with 10 mL water. The retained OTC was eluted from the cartridge with 10 mL methanol. After 1 mL 0.1% dithiothreitol was added to the eluant, the sample was concentrated to about 0.5 mL under a stream of nitrogen gas in a 40°C water bath. The volume was readjusted to 1 mL with 1M HCl and mixed using a vortex for 1 min. The suspension was filtered through a 0.45-µm filter attached to a 5-mL syringe. The filtrate was collected in a HPLC amber vial, and a 70-µL aliquot was injected onto the HPLC column.

Extraction of OMP and SDM from fish samples

The methods of Duke et al. (1993) and Weiss et al. (1987) were modified for extraction of SDM and OMP from catfish samples. To each 5-g minced fish sample (blank, augmented, and medicated) in a 50-mL centrifuge tube was added 2 mL 0.05M potassium carbonate-potassium borate-potassium hydroxide buffer (pH 10), 1 mL 1N sodium hydroxide, 400 µL 1M tetrabutylammonium hydroxide (TBAH, Fisher Scientific) in methanol, and 25 mL methylene chloride. Following manual mixing using a glass rod, the sample was vortexed at high speed for 30 sec. After the sample was centrifuged at 920 × *g* for 10 min, the supernatant was filtered through 10g anhydrous sodium sulfate packed in a funnel. The centrifuge tube and the funnel were rinsed twice with 2 mL methylene chloride. All filtrates were pooled in a 50-mL glass centrifuge tube, and the volume reduced by evaporation under a stream of nitrogen gas in a 40°C waterbath. The residue was reconstituted in 1 or 5 mL methanol and vortex-mixed for 1 min. After sitting for 5 min to allow separation of fish fat from methanol, the solvent was removed and filtered through a 0.45-µm filter. The filtrate was collected in a screw-capped glass tube, and a 0.5-mL aliquot was transferred to a HPLC vial for analysis.

HPLC determination of OTC

OTC analysis was performed on a Series 4 liquid chromatograph microprocessor-controlled solvent delivery system, an ISS-100 Intelligent Sampling System (Perkin-Elmer, Norwalk, CT) fitted with a 200 µL loop, a Waters 484 Tunable Absorbance Detector, and a Spectra-Physics integrator. The column was an ultrasphere octadecylsilane (ODS) with a particle size of 5 µm (250 mm × 4.6 mm i.d.; Beckman Instruments Inc., Fullerton, CA) and a 0.5-µm high pressure column prefilter (SSI Scientific Systems, Inc., State College, PA). Separation was achieved by isocratic elution using a mobile phase of 17 parts acetonitrile plus 83 parts of 0.02M phosphate buffer (pH 2.3) containing 1.3 mM EDTA (17:83). Prior to use, the mobile phase was filtered through a 0.45-µm filter fitted to a vacuum holder. The operating parameters were set as follows: column temperature, 30°C; flow rate, 1.0 mL/min; detector, 353 nm; and injector volume, 70 µL. The HPLC system was equilibrated for 90 min with the mobile phase until retention times and peak areas of OTC controls were reproducible.

OTC standards were analyzed together with test fish samples (blank, augmented, and treated). During analysis, a standard solution was also injected intermittently along with test samples to check chromatographic consistency. Each sample was injected twice. Peak areas of OTC standard solutions were used to prepare a standard curve. From these standard curves, OTC concentrations in test samples were calculated. At the end of each experiment, the HPLC system was flushed with methanol : water (70:30).

HPLC determination of Romet-30

The modified HPLC method of Duke et al. (1993) was applied for OMP and SDM analyses in standard solutions and fish extracts (blank, augmented, treated). The HPLC and column were the same as those for OTC analysis. However, the mobile phase was filtered acetonitrile:methanol:0.1M phosphate buffer (pH 4.0):1-heptanesulfonic acid (sodium salt) at 20:2:76:2 (v/v), at a flow rate of 1.5 mL/min, and the detector was set at 272 nm. At the end of each daily operation, the system was eluted and flushed with methanol:water mixture (70:30). Peak areas obtained from standard solutions were used to construct standard curves and linear regression equations. Sample concentrations were then calculated.

Table 3—Recoveries of ormetoprim and sulfadimethoxine from fortified catfish muscle

Fortification level (ppm)	Detected (ppm × 10^{-1})		Average recovery (%mean ± SD, n=4)		Coefficient of variation (%)	
	OMP	SDM	OMP	SDM	OMP	SDM
0.05	0.41	0.42	82.0 ± 19.0	83.5 ± 15.6	23.2	18.7
0.1	0.94	0.90	93.5 ± 10.8	89.8 ± 6.6	11.6	7.3
0.2	1.64	1.88	82.0 ± 14.8	94.1 ± 6.7	18.0	7.1
0.5	4.21	4.06	84.1 ± 7.7	83.0 ± 9.0	9.2	10.8
1.0	8.29	8.74	82.9 ± 5.6	87.4 ± 7.8	6.8	8.9
2.0	17.2	17.3	86.2 ± 6.8	86.7 ± 5.1	7.9	5.9
5.0	46.7	45.4	93.4 ± 4.6	90.8 ± 11.5	4.9	12.7
Mean			86.3 ± 5.1	87.9 ± 4.0	5.9	4.6

Table 4—Ormetoprim and sulfadimethoxine residues (ppm) in Romet-treated catfish at different feeding periods[a]

Feeding week	Romet dose (mg/kg fish)							
	Control		12.5		25		50	
	OMP	SDM	OMP	SDM	OMP	SDM	OMP	SDM
4	ND[b]	ND	ND	0.33	0.10	1.0	ND	0.12
	ND	ND	0.05	0.19	0.03	0.52	0.10	1.29
	ND	ND	ND	0.06	ND	0.09	0.11	0.12
	ND	ND	ND	0.09	ND	0.07	0.05	0.13
	ND	ND	ND	0.49	ND	0.04	0.56	2.79
	ND	ND	ND	0.04	ND	0.08	ND	ND
Avg ± S.D.	ND	ND	0.01 ± 0.02	0.20 ± 0.18	0.04 ± 0.05	0.37 ± 0.39	0.14 ± 0.21	0.74 ± 1.11
8	ND	ND	ND	0.06	0.08	0.57	ND	1.07
	ND	ND	0.05	0.71	0.07	0.35	0.39	4.26
	ND	ND	0.05	0.11	ND	0.73	0.14	1.28
	ND	ND	ND	0.75	0.07	0.82	0.26	4.05
	ND	ND	0.03	0.22	0.05	0.28	0.09	1.74
	ND	ND	0.02	ND	0.05	0.99	0.11	0.54
Avg ± S.D.	ND	ND	0.03 ± 0.02	0.31 ± 0.33	0.05 ± 0.03	0.62 ± 0.27	0.17 ± 0.14	2.16 ± 1.60

[a] Six catfish were used at each time period for each treatment group.
[b] Not detected. For the calculations of averages and standard deviations, ND was assumed to be zero.

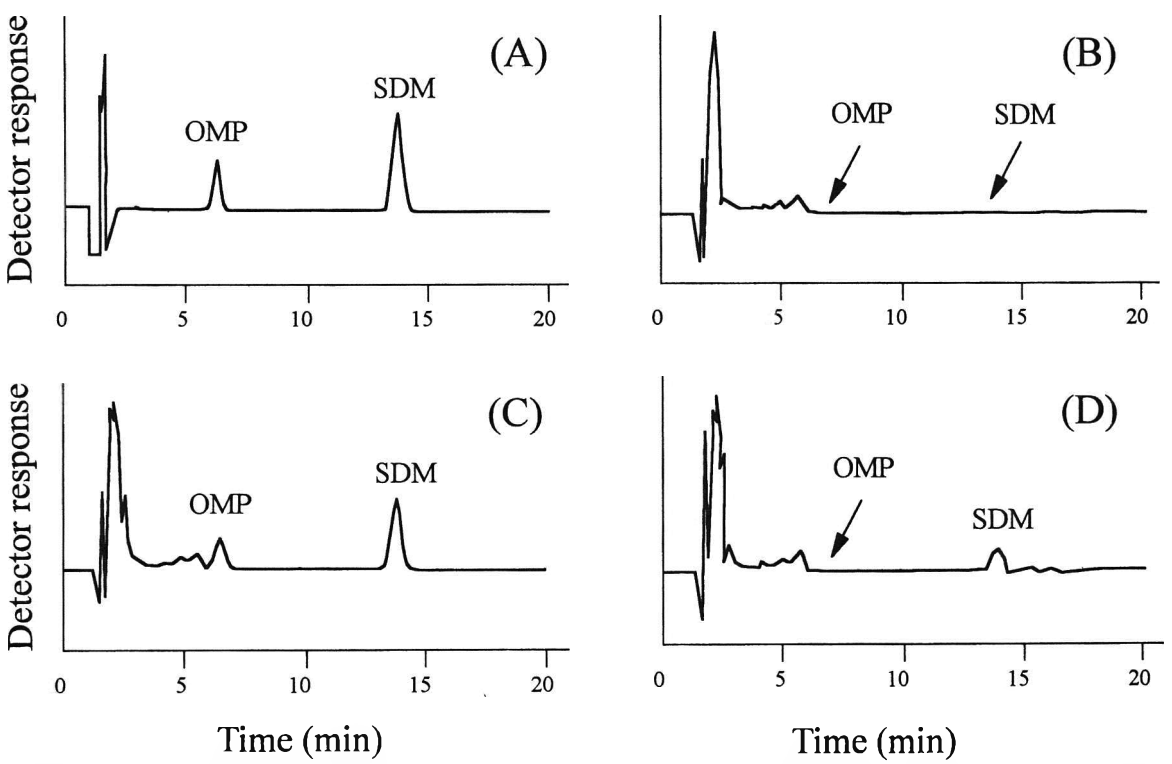

Fig. 2—Typical chromatograms of (A) a solution containing 1.0 ppm OMP and SDM, and muscle extracts of (B) an untreated catfish, (C) an untreated catfish augmented with 0.2 μg OMP and SDM/g, and (D) a catfish 24 hr after 5-day treatment with Romet-30 at 100 mg/kg body weight. HPLC conditions: see Materials & Methods.

Data for OTC, OMP and SDM residues in catfish treated with medicated feed at different dose levels were analyzed using analysis of variance (SAS Institute, Inc., 1989). PROC GLM was used in testing for differences among dose levels, feeding periods, and any interactions between dose levels and feeding periods. If no such interaction was indicated, differences among dose levels were determined at each feeding period using Tukey's HSD test procedure (Tukey, 1949).

RESULTS & DISCUSSION

HPLC determination of OTC in catfish

OTC exhibited a linear response over the range 0.25–5.0 ppm ($R^2 > 0.999$). Chromatograms of OTC standard and muscle extracts from a blank, an OTC-augmented, and an OTC-treated

Table 5—Comparisons of ormetoprim and sulfadimethoxine residues (ppm) in catfish muscle after feeding with Romet for 4 or 8 wk

Residue	Concentration (mg/kg)	Feeding weeks 4	8
OMP	12.5	0.01[a]	0.03[a]
	25.0	0.04[a]	0.05[bc]
	50.0	0.14[a]	0.17[c]
SDM	12.5	0.20[a]	0.31[a]
	25.0	0.37[a]	0.62[ab]
	50.0	0.74[a]	2.16[c]

[a-c] Within each column, means followed by the same letter are not significantly different at P = 0.05 as determined by Tukey's HSD procedure.

catfish collected from Texas A&M Univ. were compared (Fig. 1). Retention time for OTC was 6.9 min; no interfering peaks occurred with the blank or OTC-treated catfish muscle. OTC detection limits were 3.5 ng for a standard solution (50 ng/mL, 70 µL injection volume) and 0.05 ppm for catfish extracts.

The reproducibility of the HPLC method was verified by determining the intra- and inter-assay variations of OTC in fortified fillets. The average coefficient of variation (CV) for the intra-assay was 4.7%. and 6.3% for inter-assay. The recovery rate of OTC from augmented catfish was 92.5% over the concentrations of 0.05–1.0 ppm (Table 1). This was greater than the reported values of 80.9% in catfish by Long et al. (1990), 66.5% in rainbow trout by Nordlander et al. (1987), and 74.4% in chinook salmon by Aoyama et al. (1991).

OTC residues in treated catfish

Juvenile channel catfish treated with OTC or Romet-30 at Texas A&M Univ. grew rapidly during the 11-wk period (data not shown). Analyses of these fish showed that the control group contained no detectable OTC (Table 2). However, catfish fed medicated feed at different OTC levels for 4 or 8 wk contained residues. No corrections for recovery loss were made with these data. OTC residues in fish fed 12.5 mg/kg were below the 0.1 ppm tolerance level, while those in fish fed 25 mg/kg generally exceeded 0.1 ppm. OTC residues in fish fed 50 mg/kg far exceeded the 0.1 ppm tolerance level. Individual variation in OTC content also occurred in each treatment group, especially for those fish receiving high levels of OTC for 8 wk (Table 2). The differences in feed uptake and the metabolism of OTC among catfish might contribute to individual variation in residue contents. The treated catfish, when subjected to a 3-wk withdrawal period, contained no detectable OTC residue (data not shown). Apparently, OTC had been metabolized or excreted completely during the withdrawal period.

Statistical analyses showed that catfish receiving 25 or 50 mg OTC/kg for 4 wk had higher ($P < 0.05$) residues than those receiving 12.5 mg/kg. Catfish treated for 8 wk with 50 mg OTC/kg had higher ($P < 0.05$) residue level than those fed 25 mg/kg, which in turn, had higher levels than those fed 12.5 mg/kg (Table 2). Since the fish at each dosing regimen at 4 and 8 wk showed no difference in OTC residue levels, the data were pooled and compared. The dose-related increase in OTC residue for feeding between 4 to 8 wk could be expressed using a linear regression equation: residue level (ppm) = $0.0222 + 0.0033 \times$ dose (ppm) with an R^2 of 0.634.

A decrease in OTC residue in catfish following withdrawal from medicated feed also has been reported by Fribourgh et al. (1969) and Plakas et al. (1988). No OTC residue was detected in catfish muscle 48 hr after oral administration of 50, 100 or 200 mg OTC/kg of fish for 10 days (Fribourgh et al., 1969).

HPLC determination of OMP and SDM in catfish

Calibration curves comparing the peak areas and concentrations of OMP and SDM standards from seven separate trials all showed a linear relationship over the range 0.25–25.0 ppm ($R^2 > 0.999$). Representative chromatograms were compared (Fig. 2) for: (A) a solution containing SDM and OMP; (B) muscle extracts of a blank; (C) an OMP- and SDM-augmented; and (D) a Romet-treated catfish. The retention times for OMP and SDM were 7.2 and 14.5 min. No interfering peaks appeared with the blank catfish.

The instrument absolute detection limits were 6.25 ng for OMP and 2.5 ng for SDM in standard solutions. The method detection limits for OMP and SDM were both 0.05 ppm using catfish extract. A mean recovery rate of 86.3% for OMP and 87.9% for SDM was achieved from augmented catfish tissue with 0.05 to 5.0 ppm of SDM or OMP (Table 3). These values were greater than the 67.1% for OMP and 54.6% for SDM reported by Walisser et al. (1990), but lower than the 102.9% for OMP and 96.7% for SDM reported by Weiss et al. (1987).

The reproducibility of the extraction method and analytical procedure was checked using the muscle extract of Romet-treated catfish. Single-day intra-assay reproducibility was obtained with a 2.2% average CV for SDM and 3.8% for OMP. The average CV for SDM and OMP in inter-assay was 7.0 and 3.3%.

OMP and SDM residues in Romet-treated catfish

From 45 Romet-treated catfish from Auburn Univ. four contained OMP and/or SDM residue 24 hr after the last feeding of medicated feed (data not shown). One fish from the 50 mg/kg contained 0.25 ppm OMP and 0.32 ppm SDM; one from the group fed 75 mg/kg contained 0.26 ppm OMP and 0.74 ppm SDM; another from the same group contained 0.17 ppm SDM; and a fourth fish from the group fed 100 mg/kg contained 0.2 ppm SDM. These all exceeded the 0.1 ppm tolerance limit. No residue was detected in any of the catfish 48 hr or longer after the last feeding of medicated feed (data not shown). These results were in confirmation of those reported by Milner et al. (1994) in which OMP and SDM residues in catfish fillets were depleted rapidly to undetectable levels by day 2 post-treatment with 50 mg Romet-30/kg fish for 5 consecutive days.

Different levels of OMP and SDM residues were detected in Romet-treated juvenile catfish from Texas A&M Univ. (Table 4). Catfish treated with 12.5 or 25 mg Romet-30/kg for 4 or 8 wk had OMP residues below the 0.1 ppm tolerance limit. However, most of the OMP residue found in fish fed 50 mg/kg exceeded 0.1 ppm (Table 4). Unlike OMP, most of the SDM residue found in Romet-treated catfish at 4 or 8 wk exceeded 0.1 ppm. Individual variation in OMP and SDM contents was remarkable within each treatment group. The difference in drug metabolism activity among the catfish might contribute to such individual variation in residue content. No OMP or SDM was found in any of the catfish after the 3-wk withdrawal (data not shown).

Analyses of OMP and SDM residues in catfish showed that fish receiving 50 mg Romet-30/kg for 8 wk had higher ($P < 0.05$) OMP and SDM residues than those receiving 12.5 mg/kg (Table 5). Catfish receiving 25 mg Romet-30/kg for 8 wk also had higher ($P < 0.05$) OMP residue than fish fed 12.5 mg/kg. Those receiving Romet-30 for 4 wk showed no difference ($P > 0.05$) in OMP or SDM residues among the three treatment groups (Table 5).

CONCLUSION

BASED ON PUBLISHED HPLC METHODS, extraction and HPLC analyses have been developed enabling good recoveries and reproducible results in analyzing OTC residues in medicated catfish. A method employing a single extraction and a single detection wavelength also was developed to determine SDM and OMP simultaneously in Romet-treated catfish. OMP and SDM residues were found in some large catfish (≈345g) 1 day after a 5-day treatment with Romet-30 at 50-100 mg/kg body weight under natural feeding conditions. Results confirm the recommendation of the 3-day withdrawal time for Romet-30 in catfish.

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Fish Oil Dietary Effects on Fatty Acid Composition and Flavor of Channel Catfish

C.A. MORRIS, K.C. HAYNES, J.T. KEETON, and D.M. GATLIN

ABSTRACT

A typical channel catfish diet was supplemented with 0, 1.5, and 3% menhaden oil. Fillets were subjected to lipid, fatty acid, and TBA analyses. After 6 mo storage at −18°C, fillets were again analyzed for TBA value and flavor panel profile. Fillets from catfish fed diets with 1.5 and 3% supplemental fish oil had elevated levels of n-3 fatty acids ($P<0.05$). TBA number and off-flavor attributes were not affected by treatments. Menhaden oil supplementation up to 3% enhanced the content of n-3 fatty acids in channel catfish fillets without adversely affecting flavor attributes or storage quality.

Key Words: fish oil, catfish, diet supplementation, fatty acid, unsaturated fats

INTRODUCTION

CONSUMPTION OF FOODS rich in omega n-3 fatty acids such as marine fish products has increased due to research demonstrating the beneficial effects of these fatty acids in preventing certain diseases, especially cardiovascular disease (Balasubramaniam et al., 1985; Kronhout et al., 1985; Herold and Kinsella, 1986). However, not all fish products contain appreciable quantities of n-3 fatty acids (Lovell, 1988). Chanmugam et al. (1986) reported that cultured channel catfish (*Ictalurus punctatus*) fed a practical feed formulation had lower levels of n-3 fatty acids compared to wild channel catfish. This was due to the relatively low level of n-3 fatty acids in the formulated diet of cultured catfish.

Channel catfish farming is the largest aquacultural enterprise in the United States and comprises over 50% of the nation's aquaculture production by weight (USDA, 1990). Therefore, enhancing the quality of catfish products and improving their nutritional value would be of great importance. Feeds which produce excellent growth and feed efficiency in catfish in intensive (pond culture fish at > 3,000 kg/ha) culture are readily available. However, such feeds generally have not resulted in appreciable quantities of n-3 fatty acids in the processed fish (Worthington et al., 1972). Dietary changes can cause alterations in the fatty acid profile of fish fillets (Watanabe, 1982; Fair et al., 1993). The n-3 fatty acid content of channel catfish has been increased by supplementation of fish oil in prepared diets (Stickney and Andrews, 1971, 1972; Worthington and Lovell, 1973; Gatlin and Stickney, 1982). However, in most of those studies differentiation between fatty acid content of whole fish and edible tissue was limited, as was assessment of product quality through sensory attributes and shelf life.

Dupree et al. (1979) conducted sensory evaluations of catfish fed various levels of menhaden oil and corn oil and determined that even the lowest level of dietary menhaden oil (5%) resulted in lower sensory scores. Johnsen and Dupree (1991) also investigated the addition of menhaden oil (6%) into one of 21 catfish feed formulations. They found that consumer panelists found no significant differences between catfish fed varying diets. However, in a summation of five desirable attributes, trained panelists scored catfish fillets from fish fed menhaden oil the second lowest of all 21 diets.

Changes in lipid composition may affect a product's shelf life and quality during frozen storage (Stansby, 1971). Because frozen storage can affect fatty acid composition (Mustafa and Medeiros, 1985), any increases in polyunsaturation of fatty acids in catfish fillets could increase oxidative rancidity during frozen storage. Our objective was to determine whether relatively low levels of menhaden oil could be incorporated in catfish feeds to enhance the quantity of n-3 fatty acids in the fish tissue without adversely affecting flavor and storage quality.

MATERIALS & METHODS

A NUTRITIONALLY COMPLETE, practical feed formulation (Table 1), similar to those used in the commercial catfish industry was manufactured as floating pellets and served as the basal diet. Experimental treatments consisted of the basal diet supplemented with either 0, 1.5, or 3% winterized menhaden oil. Lipid fractions were sprayed on and absorbed by extruded basal diet pellets in a manner similar to that employed by commercial feed manufacturers. Supplemental lipid was added to the diets at weekly intervals and the diet ration was stored in paper feed bags at 20°C prior to feeding.

Each diet was fed in triplicate to channel catfish contained within 1-m^3 cages located in a 4-hectare reservoir. Cages were initially stocked with 250 catfish averaging 42.1 g/fish. Fish were fed the respective diets to satiation once daily for a 10-mo period. Each 2-mo period 3 fish/cage (9 fish/dietary treatment) were randomly selected and removed for analyses.

At each sampling period, 1 fillet from each fish was subjected to Folch lipid extraction (AOAC, 1990) for total lipid content. Fatty acid profiles were determined using a 10 mg aliquot of lipid extract transesterified with boron trifluoride (14%) in methanol (Morrison and Smith, 1964). Fatty acid methyl esters were analyzed with a Varian 2400 gas chromatograph (Walnut Creek, CA 94598) with a Carbowax 30 m × 0.53 mm fused silica capillary column (Supelco, Inc., Bellefonte, PA 16823). Temperatures were: injection port, 230°C; detector, 230°C; and column, 200°C. Helium was the carrier gas at linear flow of 28 cm/sec, with hydrogen and air flow of 30 and 240 mL/min, respectively. Fatty acids were identified by comparison of retention times to known standards for area percent normalization. Relative quantities were expressed as weight percent of total fatty acids in each sample.

The other fillet from each fish at sampling was subjected to thiobarbituric acid (TBA) analysis (Vyncke, 1976) and then sealed in polyeth-

Table 1—Composition of the basal diet for channel catfish (*Ictalurus punctatus*)

Ingredient	Percent as fed
Soybean meal	50.00
Corn	30.75
Wheat middlings	10.00
Menhaden fish meal	8.00
Dicalcium phosphate	1.00
Vitamin premix[a]	0.10
Trace mineral premix[b]	0.10
Coated ascorbic acid	0.05

[a] Contained (per kg of premix): vitamin A, 4,409,200 IU; vitamin D, 2,204,600 IU; vitamin E, 66,138 IU; vitamin B_{12}, 11 mg; riboflavin, 13,228 mg; niacin, 88,184 mg; d-pantothenic acid, 35,274 mg; menadione, 2,002 mg; folic acid, 2,205 mg; pyridoxine, 9,072 mg; thiamin, 10,128 mg; selenium, 0.01%.

[b] Contained (% of premix): manganese, 10; zinc, 20; Iron, 7; copper, 0.7; iodine, 0.24; cobalt, 0.01; calcium (carrier).

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Table 2—Fatty acid composition[a] of fillets from channel catfish fed diets supplemented with 0, 1.5, or 3.0% menhaden oil

Fatty acid	Percent menhaden oil in diet			P value	SEM
	0	1.5	3.0		
14:0	11.94	10.96	11.11	NS	0.423
16:0	19.56	19.58	19.50	NS	0.226
16:1ω7	7.20[b]	7.17[b]	8.07[c]	0.005	0.138
18:0	3.35	3.43	3.43	NS	0.044
18:1ω9	37.02[c]	35.89[c]	33.33[b]	0.012	0.509
18:2ω6	12.60	14.11	14.11	NS	1.05
18:3ω3	0.90[b]	1.02[b,c]	1.37[c]	0.048	0.080
18:4ω3	0.78[d]	0.62[c]	0.52[b]	0.001	0.018
20:0	0.52	0.51	0.63	NS	0.034
20:1ω9	0.82	0.81	0.80	NS	0.021
20:2ω6	0.49	0.30	0.44	NS	0.040
20:3ω6	1.11[c]	0.71[b]	0.61[b]	0.001	0.032
20:5ω3	0.64[b]	1.02[c]	1.58[d]	0.001	0.049
22:0	0.98[d]	0.63[c]	0.48[b]	0.001	0.026
22:1ω11	0.42	0.38	0.46	NS	0.040
22:5ω3	0.40[b]	0.51[c]	0.65[d]	0.001	0.020
22:6ω3	1.26[b]	2.33[c]	2.89[d]	0.001	0.094

[a] g/100g total fatty acid.
[b,c,d] Means in the same row with different superscripts are different ($P < 0.05$).

Table 3—Mean flavor profile attributes of fillets from channel catfish fed diets supplemented with 0, 1.5, or 3.0% menhaden oil

Flavor attributes[d]	Percent menhaden oil in diet			P value	SEM
	0	1.5	3.0		
Nutty/Pecan	2.76	2.88	2.67	NS	0.111
Boiled chicken/Buttery	2.65	2.69	2.66	NS	0.096
Grainy/Green vegetable	2.64	2.89	2.59	NS	0.058
Earthy/Musty	2.77	2.72	2.62	0.041	0.076
Putrid/Rancid	0.92[a]	0.81[a,b]	0.57[b]	0.004	0.057
Rotten plants	1.50	1.36	1.30	0.041	0.086
Cardboard	1.48	1.34	1.41	NS	0.062
Painty	0.25	0.29	0.22	NS	0.056
Oily/Fishy	0.27	0.44	0.48	NS	0.052
Astringent	1.61	1.68	1.48	NS	0.052
Metallic	1.40	1.59	1.42	NS	0.042
Sweet	2.04	2.02	2.08	NS	0.034
Salty	1.70	1.67	1.56	NS	0.038
Bitter	1.57	1.53	1.50	NS	0.061

[a,b,c] Means in the same row with different superscripts are different ($P < 0.05$).
[d] Attributes rated on a 0 to 15 point scale (0 indicating flavor not present).

ylene bags. Samples were stored at −20°C for 6 mo to simulate an industry storage period and then subjected to another TBA analysis and sensory evaluation (ASTM, 1968).

TBA values have been shown to be inadequate for assessing rancidity of meats (Melton, 1983). A 7-member, trained flavor profile panel was used to determine the presence or absence of off-flavors. Panel members participated in 15 training sessions to become familiar with off-flavors commonly found in channel catfish before receiving experimental samples. The training sessions were based upon a lexicon of off-flavors compiled by Johnsen et al. (1987). During each evaluation, fillets from three fish/cage were combined and homogenized while partially frozen. Samples were formed into 50-g balls, cooked in a microwave oven at maximum power for 3 min and served hot to each panelist. One sample/diet for a total of three samples/session was served to individual panelists seated in partitioned booths with controlled lighting and negligible background disturbance from noise. Samples were served in varying order so that samples from the same diet were not served in the same order more than once. Panelists participated in three sessions/day for a total of nine samples/day. Both distilled and carbonated water were supplied for panelists to cleanse their palates between samples. Sensory attributes evaluated were: nutty/pecan, boiled chicken/buttery, grainy/green vegetable, earthy/musty, putrid/rancid, rotten plants, cardboard, painty, oily/fishy, astringent, metallic, sweet, salty, and bitter. Each sample was rated on a scale of 0 to 15 for each attribute with 0 indicating absence of the attribute.

All analytical data were subjected to analysis of variance blocking by cage, diet, slaughter age, and diet by slaughter age interaction. Means were separated using Duncan's multiple range test in the Statistical Analysis System (SAS Institute, Inc., 1986). Differences were considered significant at $P < 0.05$.

RESULTS & DISCUSSION

THERE WERE NO SIGNIFICANT diet by harvest age interactions for the major fatty acids or sensory panel attributes, therefore, marginal means were reported across harvest ages in tables. Compositional analysis of channel catfish fillets indicated that total lipid was affected ($P < 0.05$) by dietary menhaden oil supplementation. The mean fat contents for the respective diets were: control, 3.80% (SE = 0.09%); 1.5% supplemental oil diet, 4.02% (SE = 0.08%); and 3.0% supplemental oil diet was 4.34% (SE = 0.08%). Mean separation showed that fish fed the highest level of supplemental menhaden oil deposited more fat than those fed the control diet ($P < 0.05$). Lipid levels we observed were similar to those previously reported in channel catfish (USDA, 1986a), although higher levels have been reported (Lovell, 1988; Huang et al., 1994). This may be influenced by fish size as well as dietary energy and lipid content (Gatlin et al., 1986; Nettleton et al., 1990).

The fatty acid profiles of catfish fillets were also compared (Table 2). The fatty acid profile of fish fed the control diet was similar to that reported by Nettleton et al. (1990). Changes in fatty acid composition and specifically increases in eicosapentaenoic (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) were observed in fillets as a response to increased dietary menhaden oil supplementation. Increases in EPA and DHA observed in the supplementary menhaden oil treatments could be attributed to the high percentage of EPA and DHA in the menhaden oil we used (24.56% EPA+DHA). In addition to increases in EPA and DHA, docosapentaenoic acid (22:5ω3) levels increased in fillets from fresh fish fed supplemental menhaden oil (Table 2). Other notable changes in the fatty acid composition included increases in palmitoleic (16:1ω7) and linolenic (18:3) fatty acids and corresponding declines in oleic (18:1ω9), 18:4ω3, 20:3ω6, and behenic (22:0) fatty acids. Lovell (1988) reported an increase in n-3 fatty acids of channel catfish fillets with dietary menhaden oil supplementation; however, detailed information concerning fatty acid composition, frozen storage quality, and sensory evaluation was not presented. Although the n-3 fatty acid content of catfish fillets increased in our study to levels comparable to those in wild channel catfish (Chanmugam et al., 1986), the overall quantity of these fatty acids was relatively low compared to marine species such as mackerel (USDA, 1986a, b). However, the total lipid content in channel catfish is generally much lower than many of the "fattier" marine species.

Although the n-3 fatty acid content of catfish fillets increased with dietary supplementation of menhaden oil, quality of the product in frozen storage was not impaired based on TBA values. Diet did not have an effect ($P > 0.05$) on TBA values. There was a general increase in TBA value with the 6-mo frozen storage period, but all values remained below 1 TBA, regardless of dietary treatment or storage time, indicating acceptable product quality. Data concerning this specific aspect of product quality have not been presented in other studies investigating enhancement of n-3 fatty acids (Lovell, 1988).

Supplemental fish oil in the diet also did not adversely affect the flavor of channel catfish based on the trained flavor panel profile (Table 3). Some off-flavors were detected at relatively low levels in certain samples. However, in most cases, dietary menhaden oil supplementation either had no effect on, or significantly improved sensory scores. The fishy flavor that has been noted in other studies (Lovell, 1988), where higher levels of supplemental menhaden oil were fed, was not detectable in our study. This was likely due to either the relatively low levels of menhaden oil, higher-quality raw materials, or addition of 66 IU vitamin E/kg diet. Research by Bai and Gatlin (1993) and Gatlin et al. (1992) showed that supplemental vitamin E had no effect on fish growth, feed efficiency or proximate composition of whole-body and fillet tissues, but provided additional protection against lipid oxidation during frozen storage.

Based on our results, the content of n-3 fatty acids could be increased in channel catfish fillets without adversely affecting frozen-storage quality and sensory attributes. Significant enhancement of n-3 fatty acids in channel catfish was achieved while maintaining relatively low lipid levels. However, those n-

3 fatty acid levels were generally much lower than present in high-lipid marine fish.

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This research was funded in part by a grant from the United States Department of Agriculture and the Texas Agricultural Experiment Station under project H-6556. We thank Zapata Haynie Corporation, Reedville, VA, for providing the menhaden oil.

*ANTIBIOTIC RESIDUES IN CHANNEL CATFISH. . .*From page 1224

The 21-day withdrawal period for OTC in catfish appears suitable even for fingerling fish receiving OTC treatment longer than 10 days.

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Ms received 7/6/95; revised 8/21/95; accepted 8/25/95.

Florida Agricultural Experiment Station Journal Series No. R-04600.

This research was supported in part by the Southern Regional Aquaculture Center through a grant from the United States Department of Agriculture.

Activity Staining of Pacific Whiting (*Merluccius productus*) Protease

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ABSTRACT

An activity staining method using SDS-substrate gel was used to determine Pacific whiting proteolytic activity. The most active protease band was resolved at an apparent M_r of 39,500 corresponding to the activity of cathepsin L. The activity was more pronounced for ocean-caught Pacific whiting than Puget Sound Pacific whiting or ocean-caught arrowtooth flounder. Gel electrophoresis activity staining of proteolytic enzymes in Pacific whiting was not as sensitive as the TCA-azocasein assay but provided information on the molecular properties of the enzyme and a semi-quantitative estimation of activity.

Key Words: azocasein, protease, staining, whiting

INTRODUCTION

NUMEROUS ABUNDANT FISH SPECIES, i.e., Pacific whiting (*Merluccius productus*), arrowtooth flounder (*Atheresthes stomias*), Atlantic croaker (*Micropogon undulatus*), white croaker (*Siena schlegeli*), and mackerel (*Scomber australasicus*), have not been utilized to their full value due to protease-induced soft texture. Several types of proteases, i.e., serine proteases (Busconi et al., 1989; Martone et al., 1991; Toyohara and Shimizu, 1988), alkaline proteases (Lin and Lanier, 1989; Makinodan et al., 1985, 1987; Stoknes et al., 1993; Su et al., 1981), cathepsins (Jiang et al., 1990, 1992; Masaki et al., 1993; Seymour et al., 1994; Yamashita and Konagaya, 1990, 1991), and calpains (Jiang et al., 1991; Tsuchiya and Seki, 1991; Wang et al., 1993), have been reported to contribute to postmortem softening of fish muscle. Among these, cathepsin L, a lysosomal protease, has been shown to be very important in tissue degradation of Pacific whiting surimi at elevated temperatures often used in surimi manufacture (An et al., 1994a; Chang-Lee et al., 1989).

Studies of the contribution of various proteases to tissue softening have been hampered by the lack of specific protease substrates or by similarities in enzyme properties which make it difficult to distinguish activity of a specific protease. Comparison of activities by gel electrophoresis can facilitate such biochemical studies. Activity staining of SDS-substrate gels is a method of choice for identification and characterization of specific proteases in complex systems such as fish muscle. Activity staining of protease/protease inhibitor activities in electrophoresed gels has been carried out utilizing fluorogenic substrates (Mason et al., 1984), chromophore development (Kollipara and Hymowitz, 1992), and protein substrates (Bertolini and Rohovec, 1992; García-Carreño et al., 1993; Lacks and Springhorn, 1980; Pike et al., 1992). Among these, the staining method based on protein substrates, though less specific, can provide general information on a wide range of proteases. García-Carreño et al. (1993) developed a protease activity staining method for trypsin using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) substrate gels. The method utilizes denaturing conditions, which are advantageous for determining the molecular weight of proteins in addition to semi-quantitative measurement of specific isozymes. Proteases, particularly those of a single polypeptide, were reported to renature rapidly upon removal of SDS from the gel and could be detected by clear zones on a dark background corresponding to activity (Lacks and Springhorn, 1980). Our objective was to apply a protease activity staining method for Pacific whiting protease and to compare and quantify proteolytic activities in Pacific whiting.

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MATERIALS & METHODS

Fish source

Pacific whiting and arrowtooth flounder caught off the Oregon coast were off-loaded at local processors within 24 hr of harvest. Pacific whiting from Puget Sound was obtained from Crystal Ocean Seafood (Burlington, WA). Whole fish were transported in ice to the Oregon State University Seafood Laboratory in Astoria and filleted immediately. A portion of the fillets were used fresh, while the rest were vacuum-packed, frozen in a blast freezer at −30°C and stored at −20°C until used.

Preparation of fish juice and heat-treatment

Sarcoplasmic fluids of fish muscle were prepared according to the method of Erickson et al. (1983). Frozen fish fillets were thawed, finely chopped, and then centrifuged at 3,000 × *g* for 30 min to collect supernatant as an enzyme source. The supernatant was termed "fish juice" (FJ).

FJ was heat-treated at 60°C for 3 min and centrifuged at 7,800 × *g* for 15 min. Seymour et al. (1994) showed that heat treatment at 60°C concentrated the enzyme activity fourfold. The collected supernatant was referred to as "heat-treated fish juice" (HJ) and used for activity staining of Pacific whiting and arrowtooth flounder proteases on SDS-substrate gels.

Purification of cathepsin L

Cathepsin L was purified from parasitized Pacific whiting to electrophoretic homogeneity by the method of Seymour et al. (1994) using: heat treatment and chromatography on butyl-Sepharose (Pharmacia Biotech Inc., Piscataway, NJ), diethylaminoethyl (DEAE) Bio-Gel A (Bio-Rad Laboratories, Richmond, CA), and Superose-12 (Pharmacia Biotech Inc., Piscataway, NJ). Activity was analyzed by TCA-azocasein assay as described in the following section, to screen for cathepsin L activity during purification.

Enzyme assay

Protease activity was assayed by the TCA-azocasein method (An et al., 1994b) for FJ, HJ or purified enzyme. Activity of FJ and HJ was analyzed for 30 min, and that of purified enzyme for 5 to 10 min at 55°C in McIlvaine's buffer, pH 5.5, containing 2 mM β-mercaptoethanol (βME) with azocasein as substrate. Blanks were prepared in the same manner, except that enzyme was added after addition of 50% trichloroacetic acid (TCA). Activity was determined by an increase in absorbance at 450 nm compared with that of the blank and expressed as ΔA_{450}.

Protein determination

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

SDS-substrate and SDS-PAGE gels

SDS-substrate gels (13% T) containing 0.03% casein were prepared according to the method of Garcia-Carreño et al. (1993). HJ (200 μg) or purified Pacific whiting cathepsin L (25 μg) was mixed with sample

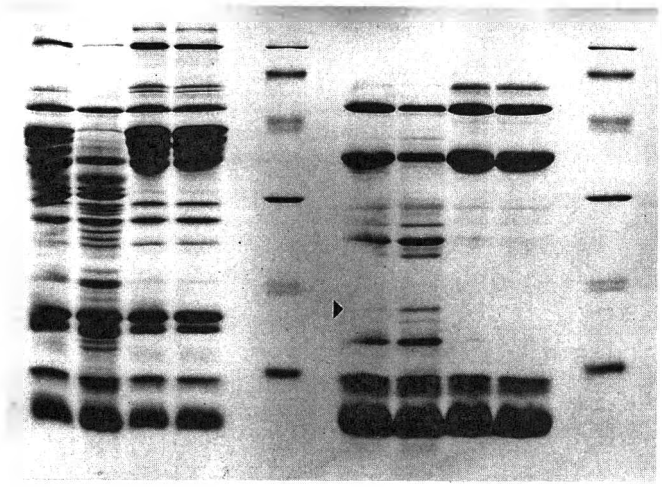

Fig. 1—Effects of reducing agent and boiling on banding patterns of Pacific whiting fish juice (A) and heat-treated fish juice (B) on SDS-PAGE. Proteins were subjected to sample treatments with βME or boiling prior to electrophoresis run, analyzed on 15% SDS-PAGE gel, and stained by Coomassie blue R-250. Protein samples were (1) control, (2) with 2.5% βME, (3) boiled for 1.5 min, and (4) boiled for 1.5 min with 2.5% βME. The marked arrow indicates cathepsin L. MW indicates molecular weight protein standards with M_r 94,000; 67,000; 43,000; and 30,000 from the top.

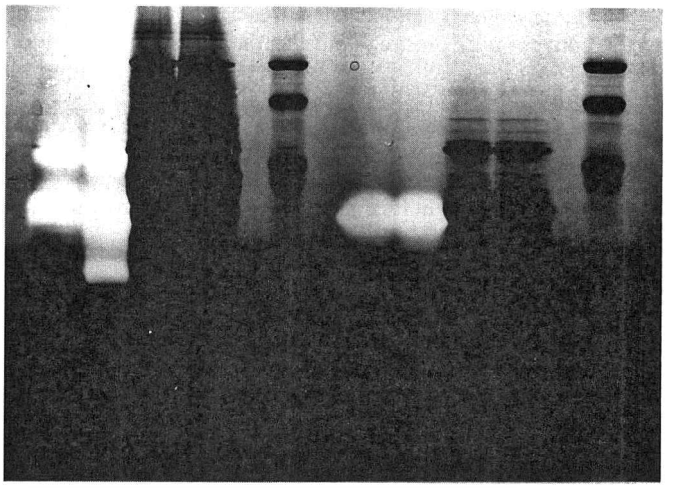

Fig. 2—Effects of a reducing agent and boiling on banding patterns of Pacific whiting fish juice (A) and heat-treated fish juice (B) on SDS-substrate gel stained for proteolytic activity. Proteins were subjected to sample treatments with βME or boiling prior to electrophoresis run, analyzed on 12% SDS-substrate gel, and stained for proteolytic activity. Protein samples were (1) control, (2) with 2.5% βME, (3) boiled for 1.5 min, and (4) boiled for 1.5 min with 2.5% βME. MW indicates visible molecular weight protein standards with M_r of 94,000; 67,000; and 43,000 from the top.

buffer (Laemmli, 1970) containing no βME at 3:1 (v/v) ratio and loaded directly on the substrate gels, unless otherwise specified. After separation of proteins by electrophoresis (Protein II, Bio Rad Laboratories Inc., Richmond, CA), gels were stained for activity with the modified method of Garcia-Carreño et al. (1993).

SDS-PAGE gels (13 or 15% T) were also prepared according to the method of Laemmli (1970). Composition of SDS-PAGE gels were essentially the same as that of SDS-substrate gels except that no casein was incorporated into SDS-PAGE gels. Protein samples were boiled for 1.5 min in the sample treatment buffer with or without 2.5% βME as specified and applied on the gels.

Electrophoresis

Gels were electrophoresed at a constant current of 10 mA per slab for 16 to 18 hr for activity staining or 30 mA per slab for total protein staining with 0.125% Coomassie brilliant blue R-250. All SDS-PAGE reagents were of electrophoresis purity. Low-molecular-weight standards (Pharmacia, Piscataway, NJ) included phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400).

Activity zone development and gel staining

SDS-substrate gels were stained for proteolytic activity. After electrophoresis, gels were incubated in 2.5% Triton X-100 with agitation for 1 hr at room temperature with one change of solution. Gels were transferred to McIlvaine's buffer, pH 5.5 (0.2M phosphate and 0.1M citrate), and incubated for 1 hr at 55°C for activity development. They were then stained in 0.125% Coomassie brilliant blue R-250 and destained in 30% ethanol and 10% acetic acid. The development of a clear zone on the blue background indicated proteolytic activity. For SDS-PAGE, gels were fixed and stained in 0.125% Coomassie brilliant blue R-250 immediately after electrophoresis.

Effect of inhibitors

HJ was incubated with an equal volume of chemical inhibitors or food-grade protease inhibitors for 15 min at room temperature prior to electrophoresis. Chemical inhibitors included 0.01 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.01 mM pepstatin A (PS), and 1 mM EDTA. The inhibitor-treated HJ was applied onto SDS-substrate gel and stained for protease activity.

RESULTS & DISCUSSION

Effects of βME and heat treatment

Effects of sample treatments on migration of proteins and banding patterns were compared in SDS-PAGE gels (Fig. 1). Both sample boiling and addition of βME affected protein banding patterns of FJ and HJ. Native FJ proteins, not boiled before electrophoresis, showed 17 visible bands. When βME was added, bands increased to 23. The bands were resolved at the lower molecular weight region by dissociation of protein subunits which increased the protein migration rate in the gel. When FJ was boiled, fewer protein bands were visible than in native FJ. The protein bands in the range of M_r 40,000 to 50,000 were mostly missing. With boiled samples, no obvious change was made by addition of βME.

Native HJ showed only seven protein bands, much fewer than those of FJ (Fig. 1). By addition of βME to the native HJ three additional minor protein bands appeared within the region between M_r 30,000 and 43,000. The band corresponding to cathepsin L on the activity staining condition was also detected in that sample and is marked by an arrow (Lane B2). Boiled samples in the presence or absence of βME showed the least protein bands, mostly lacking in the region between M_r 30,000 and 43,000.

Proteolytic activities of FJ and HJ were analyzed on the SDS-substrate gel (Fig. 2) by the same sample treatments as used for Fig. 1. Two clear zones were observed for FJ at M_r 54,200 and 39,500, and one additional clearing at M_r 31,700, when 10 mM βME was added to FJ. The clearing at M_r 31,700 is probably a cysteine protease since it was activated by addition of βME to the extract (Asghar and Bhatti, 1987). All proteolytic activities were completely eliminated, with no clearing on the gel, when samples were boiled prior to electrophoresis.

HJ usually showed one clear zone at M_r 39,500; possibly through the activity of cathepsin L on the SDS-substrate gel. HJ showed two additional minor clearings at M_r 54,200 and 31,700, when samples had extremely high activity. The clearing at M_r 31,700 was postulated to be due to completely reduced cathepsin L, while the activity at M_r 54,200 has not been identified. No significant effect of βME was observed on the number of clear zones, indicating the proteolytic activity in HJ was mainly af-

fected by the original activity in the muscle extract rather than by the activation of protease. Due to the higher specificity of HJ for cathepsin L activity, HJ was prepared as a source of enzyme for further studies.

Identification of cathepsin L activity

The molecular weight of Pacific whiting cathepsin L was reported to be M_r 28,800 by SDS-PAGE (Seymour et al., 1994). However, when purified Pacific whiting cathepsin L was used for activity staining, one clear zone appeared at M_r 39,500 (Fig. 3) corresponding to the clear zone observed with Pacific whiting HJ. To clarify the difference in molecular weights, purified Pacific whiting cathepsin L was applied on the SDS-PAGE with and without heat treatment or addition of βME.

Heat treatment was the main factor affecting migration rate of purified cathepsin L (Fig. 4). The protease, which was not heat-treated prior to SDS-PAGE, was resolved at an apparent M_r of 39,500. The heat-treated sample in the absence or presence of βME was observed at an apparent M_r of 31,700. Addition of βME did not change the migration rate of purified cathepsin L, once heat-denatured. The difference in migration rate of heat-treated and nonheat-treated purified cathepsin L was hypothesized to be caused by internal disulfide bonds in the protein. Ovotransferrin, a glycoprotein in egg white, contains multiple disulfide bridges that are internal and are not reduced by βME without prior denaturation (Stevens, 1991). The presence of a carbohydrate moiety could also influence migration rates. Carbohydrates on proteins were reported to reduce the ratio of SDS binding to the mass of proteins and thus retard the migration rate during electrophoresis. This would result in an increase in apparent molecular weight of proteins (Segrest and Jackson, 1972). About 90% of cathepsin L from mackerel was reported to be glycosylated and adsorbed onto concanavalin A-Sepharose resin (Lee et al., 1993). However, both glycosylated and nonglycosylated cathepsin L had similar specific activities (Mason et al., 1984).

The clearing of SDS-substrate gel by the activity of cathepsin L was confirmed by treatment with chemical inhibitors (Fig. 5). E-64, a specific inhibitor for cysteine protease (Asghar and Bhatti, 1987), completely eliminated clearing on SDS-substrate gels. PMSF, a serine protease inhibitor, and pepstatin, an aspartic acid protease inhibitor, reduced activity slightly, possibly by modification of amino acid side chains other than the active site (Glazer et al., 1975). EDTA, a metalloprotease inhibitor, did not affect the activity. These data verified that the clearing at M_r 39,500 was due to a cysteine protease.

Comparison of activity level in fish muscle

Proteolytic activity of Pacific whiting from the Oregon coast as well as from Puget Sound, WA, and arrowtooth flounder from the Oregon coast was examined on SDS-substrate gel (Fig. 6). Pacific whiting from the Oregon coast showed extremely high proteolytic activity. In addition to the main clearing at M_r 39,500, corresponding to the activity of native Pacific whiting cathepsin L, two minor bands at M_r 54,200 and 31,700 were detected. For arrowtooth flounder, only a weak clearing at M_r of 39,500 was detected, indicating lower cathepsin L activity than in Pacific whiting. Preliminary results have shown that the proteolytic activity of arrowtooth flounder was largely associated with alkaline proteases (An et al., 1995). Possible activity of alkaline proteases, which are composed of nonidentical subunits, was not detected with this method. Oligomeric enzymes composed of nonidentical subunits do not renature in SDS-substrate gels after the disruption of disulfide bonds and separation of subunits (Lacks and Springhorn, 1980).

The difference in cathepsin L activity may also be explained by the infecting Kudoa species, *Kudoa paniformis* and *K. thyrsitis* which have been identified in Pacific whiting muscle (Patashnik et al., 1982), while *K. thyrsitis* has been tentatively identified from arrowtooth flounder (Greene and Babbitt, 1990). Of these two species, *K. paniformis* has been reported to be more virulent and has more severe effects on whiting flesh texture (Patashnik et al., 1982). This could explain higher cathepsin L activity in Pacific whiting. Greene and Babbitt (1990) reported no visible signs of muscle abnormalities in arrowtooth flounder, in contrast to the visible pseudocysts in Pacific whiting.

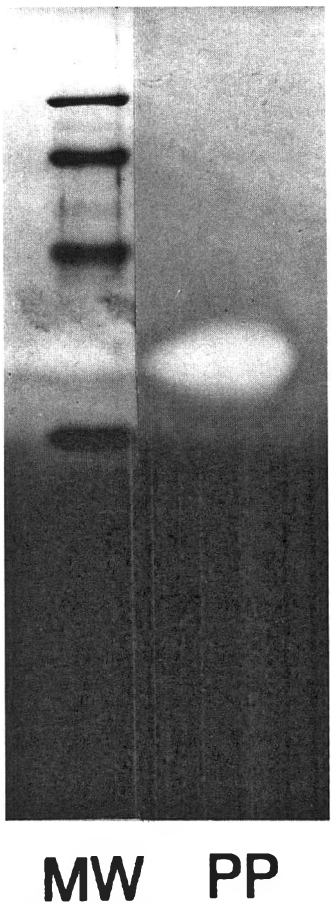

Fig. 3—Activity staining of purified cathepsin L from Pacific whiting on SDS-substrate gel. MW indicates visible molecular weight protein standards with M_r 94,000; 67,000; 43,000; and 30,000 from the top.

Pacific whiting from Puget Sound is not infected with Kudoa and is considered to be a separate stock from that of the U.S. northwest coast (Pedersen, 1985). Puget Sound Pacific whiting applied on the SDS-substrate gel did not show any clearing, indicating negligible proteolytic activity in the muscle.

Quantification of proteolytic activity

Activity staining of SDS-substrate gel was evaluated as a quantification method of proteolytic activity, and its sensitivity was compared with TCA-azocasein assay. Pacific whiting muscle infected with Myxosporidia had increased levels of proteolytic activities (Morrissey et al., 1995). Therefore, HJ was prepared from infected muscle with high levels of black pseudocysts (as inspected visually). The HJ showing proteolytic activities ranging from ΔA_{450} 0.94 to 2.57 were subjected to activity staining of SDS-substrate gel.

Large visible clearings were observed with HJ in lanes 4, 1, and 2; activity was determined to be ΔA450 2.57, 2.39, and 2.39, respectively (Fig. 7). Lane 3 with ΔA450 2.10 also showed a readily recognizable clear zone. However, HJ used for lanes 5 and 6, with activity ΔA_{450} 1.28 and 0.94, respectively, did not show any clearings. In general, clearing at M_r 39,500 by cathepsin L was not detectable by activity staining when sample activity was lower than ΔA_{450} 1.28. Thus, activity staining of

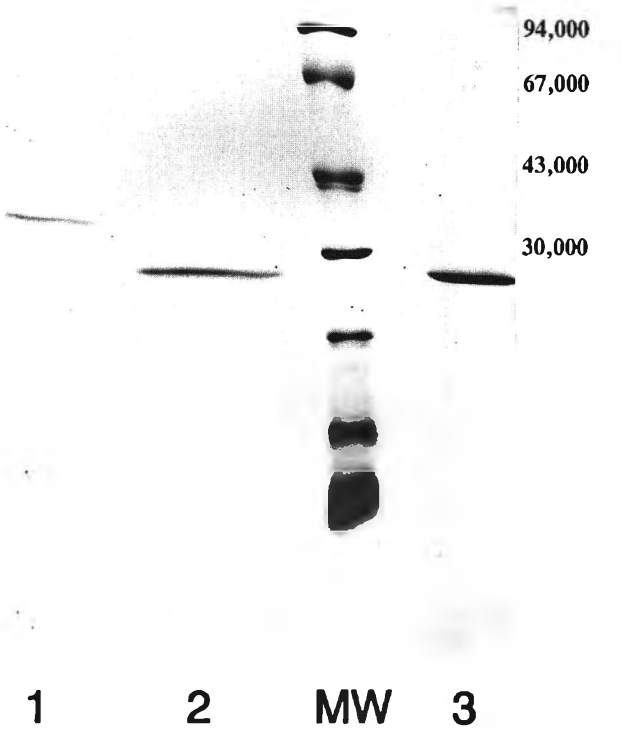

Fig. 4—Effects of a reducing agent and boiling on banding patterns of purified cathepsin L from Pacific whiting on SDS-PAGE. Proteins were subjected to sample treatments with βME or boiling prior to electrophoresis run, analyzed on 15% SDS-PAGE gel, and stained by Coomassie blue R-250. Protein samples were (1) control, (2) boiled, and (3) boiled for 1.5 min with 2.5% βME. MW indicates molecular weight protein standards with M_r of 94,000; 67,000; 43,000; 30,000; 20,100; and 14,400 from the top.

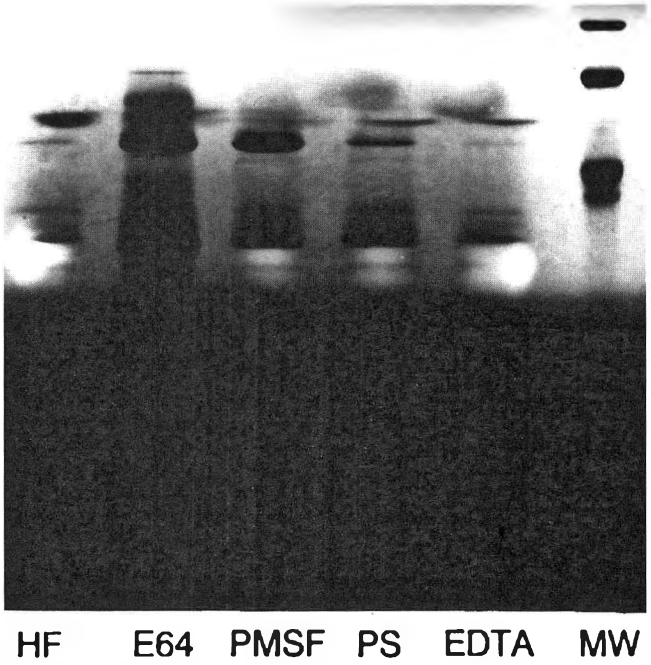

Fig. 5—Effect of chemical inhibitors on proteolytic activity of Pacific whiting. HJ were treated with no inhibitors (HF), 0.01 mM E–64 (E64), 1 mM PMSF (PMSF), 0.01 mM pepstatin A (PS), and 1 mM EDTA (EDTA). MW indicates visible molecular weight protein standards with M_r 94,000; 67,000; and 43,000 from the top.

SDS-substrate gel was less sensitive than TCA-azocasein assay and could only detect high-level proteolytic activities in muscle extracts. This indicated that it may serve as a semi-quantitative method for estimation or detection of proteolytic activity in the

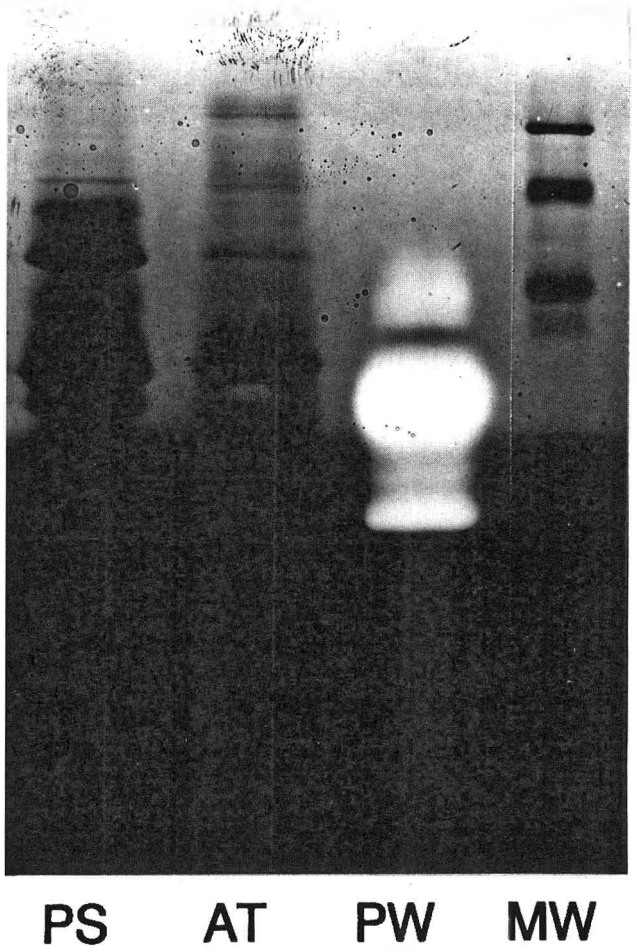

Fig. 6—Comparison of proteolytic activity of various fish species by activity staining. PS, Pacific whiting from Puget sound; AF, arrowtooth flounder; and PW, Pacific whiting from Oregon coast. MW indicates visible molecular weight protein standards with M_r 94,000; 67,000; and 43,000 from the top.

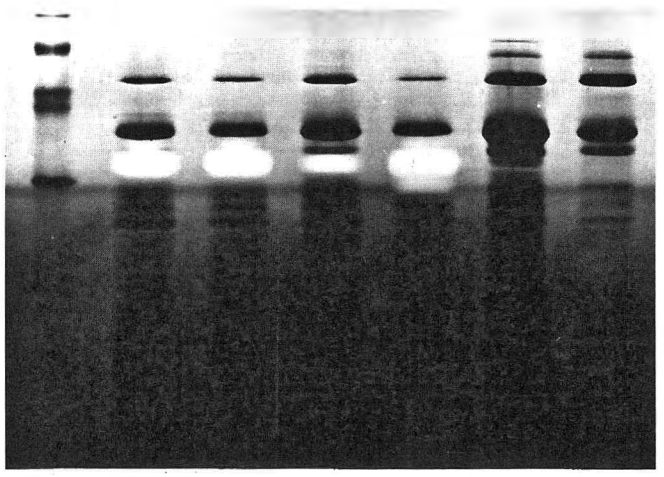

Fig. 7—Comparison of activity staining method with TCA azocasein assay for quantitation of proteolytic activity. HJ with various levels of proteolytic activities were prepared from selected Pacific whiting muscles with visible black pseudocysts, and their proteolytic activities were determined by TCA-azocasein assay: lane 1, ΔA_{450} 2.39; lane 2, ΔA_{450} 2.39; lane 3, ΔA_{450} 2.10; lane 4, ΔA_{450} 2.57; lane 5, ΔA_{450} 1.28; and lane 6, ΔA_{450} 0.94. MW indicates visible molecular weight protein standards with M_r 94,000; 67,000; 43,000; and 30,000 from the top.

muscle extract. However, this method provided valuable information on molecular characteristics of protease in the complex muscle system, which could not be easily obtained by other methods.

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Ms received 3/29/95; revised 5/23/95; accepted 5/30/95.

This research was supported in part by Oregon Sea Grant with funds from NOAA, Office of Sea Grant, U.S. Dept. of Commerce, under Grant No. NA36RG0451 (Project No. R/SF-1); U.S. Dept. of Agriculture under Grant No. 93-34307-8371. The U.S. Government is authorized to produce and distribute reprints for governmental purposes, notwithstanding any copyright notation that may appear hereon.

Renaturation of α1 Chains from Shark Skin Collagen Type I

YOSHIHIRO NOMURA, MASAYA YAMANO, and KUNIO SHIRAI

ABSTRACT

Renaturation of α1 chains from shark skin collagen (I) was measured by the increase in optical rotation and viscosity. The rotation change of the α1(I) chains became constant after 18 hr, while the viscosity increased further with time. Size exclusion chromatographic patterns of renaturation products of α1(I) chain gave similar results to that of native collagen. The renaturation products remelted with rising temperature in a similar way to the melting of native collagen as judged from the rotation-temperature curve.

Key Words: renaturation, collagen, gelatin, shark, optical rotation

INTRODUCTION

THE MOLECULAR STRUCTURE and biological functions of collagen type I from land animals have been extensively investigated. Collagen type I, especially bovine skin collagen, has been utilized in foods, cosmetics and medical materials (Nimni, 1988). For collagen type I from aquatic animals, there are limited studies, mainly in comparative biochemistry (Kimura et al., 1981; Kimura and Ohno, 1987; Kelly et al., 1988). Few papers have suggested a possibility of fish gelatins for food and photographic materials (Berg et al., 1985; Fraga and Williams, 1985; Leuenberger, 1991). Collagen type I from aquatic animals may provide an alternate collagen source, containing fewer imino acid residues and with a lower denaturation temperature than collagen from land vertebrates (Kimura and Ohno, 1987; Piez, 1988; Kelly et al., 1988). More information is needed on the structural features and utility of aquatic animal collagen. The among type I collagens from aquatic animals, shark skin collagen (I), in particular, is potentially important. Shark fish are collected in conjunction with tuna fishery and the meat and skin serve as food materials. Shark skin collagen (I) is unique in that its α component is obtained exclusively in the form of the α1 chain. Shark skin α1 chain gelatin may have useful and different functions from gelatins of land animals which are complicated mixtures of α1 and α2.

Collagen is denatured to gelatin by heating such that its rigid helix structure becomes a flexible random coil. The denatured gelatin partially recovers the features of native collagen if it is kept below its denaturation temperature (Veis and Cohen, 1960; Piez and Carrillo, 1964; Nishihara and Miyata, 1964). This phenomenon is known as renaturation. The recovery influences the rheology of the gelatin gel and is important when gelatin is used as a gel in foods. Our objective was to study renaturation properties of collagen (I) from shark skin.

MATERIALS & METHODS

Isolation of shark skin α1(I)

Collagen type I was prepared from fresh great blue shark (*Prionace glauca*) skin, according to the method of Kimura et al. (1981). α1(I) was purified by the use of carboxymethyl (CM)-chromatography and size exclusion chromatography (SEC).

First step purification of α1(I) was done by CM-chromatography as follows: supporting gel, CM-Toyopearl 650S (TOSHO, Tokyo); column size, 30 mm i.d. and 400 mm length; elution, linear gradient of sodium chloride from 0.05 to 0.25M in 0.06 M acetate buffer at pH 4.8 containing 1M urea; flow rate, 120 mL/hr; temperature, 40°C; detection, absorbance at 230 nm; fraction, 8 mL/tube.

Final purification of α1(I) was done by SEC as follows: supporting column, a series combination of Asahipak GS-520FP, GS-620FP, GS-720FP (Asahikasei, Tokyo); column size, 21.5 mm i.d. and 300 mm length each; mobile phase, 0.1M sodium phosphate buffer at pH 7.0 containing 2M urea; flow rate, 1.0 mL/min; temperature, 40°C; detection, absorbance at 230 nm.

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Measurement of renaturation process with specific rotation

The renaturation process of α1(I) was measured by specific rotation. The rotation of α1(I) was measured as follows: α1(I) was dissolved by heating at 40°C for 10 min in 0.01M sodium phosphate buffer at pH 7.0 to give a concentration of about 2 mg/mL. After filtration through a 0.45 μ-pore-size membrane (DISMIC-25cs, Advantec, Tokyo), the specific rotation of the sample was measured with a polarimeter (SEPA-200, digital type, Horiba, Tokyo) at 589 nm at 10°C. The specific rotation was calculated according to the following equation:

$$[\text{specific rotation}] = [\text{rotation (deg)}]/[\text{cell length (dm)}] \times \text{concentration (g/mL)}]$$

Percent recovery, $[X_H(\%)]$ was calculated by the following expression:

$$X_H(\%) = ([\alpha]_t - [\alpha]_{gelatin})/([\alpha]_{collagen} - [\alpha]_{gelatin})$$

where $[\alpha]_t$, specific rotation of sample at time t; $[\alpha]_{collagen}$, specific rotation of collagen; $[\alpha]_{gelatin}$, initial specific rotation of sample (gelatin).

The denaturation temperature of shark skin collagen (I), and the redenaturation temperature of renatured α1(I) and shark skin collagen (I) were measured by specific rotation. Shark skin collagen (I) was dissolved in 0.01M acetic acid to give a concentration of about 2 mg/mL. After centrifugation (model 50A-6, Sakuma, Tokyo) at 18,000 rpm (36,000 × *g*), the specific rotation of the sample was measured as the temperature was raised from 10°C to 40°C in 4 hr.

The protein concentration was measured by the microbiuret method (Itzhaki and Gill, 1964).

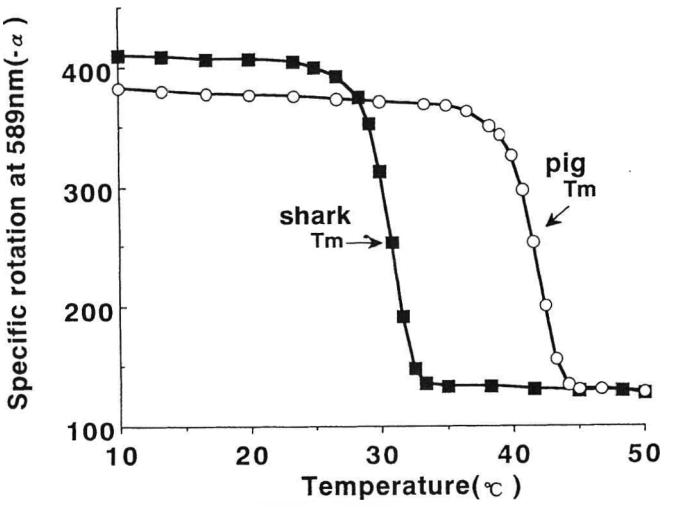

Fig. 1—Denaturation curves of shark and pig skin collagen (I) as measured by specific rotation.

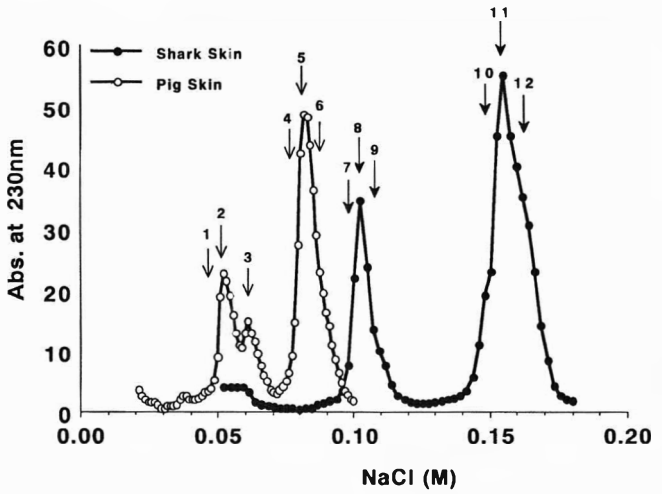

Fig. 2—CM-chromatographic patterns of denatured shark and pig skin collagens. 1–12, samples examined by SDS-PAGE (Fig. 3).

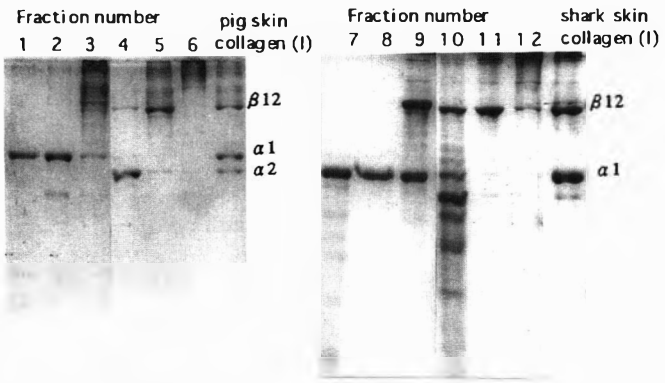

Fig. 3—SDS-PAGE patterns of the major fractions from CM-chromatography of shark and pig skin collagen (I). 1–6, Fraction number from pig skin collagen (I); 7–12, Fraction number from shark skin collagen (I).

Measurement of viscosity

Specific viscosity was measured with an Ostward capillary viscometer (t_0 = 150–200 sec, Kaburagi, Tokyo), and reduced viscosity was extrapolated to 0 concentration to obtain the intrinsic viscosity. The solvent was 0.01M sodium phosphate buffer at pH 7.0. The denatured and renatured states of α1(I) chain were measured at 40°C and 10°C, respectively.

Aggregation of renatured products

The renatured collagen-like chains were separated from the renaturation system by salting-out at a final concentration of 5% sodium chloride in 0.5M acetic acid after standing overnight. After centrifugation at 18,000 rpm, the collagen-like chains were dissolved in SEC solvent and applied to the column.

Aggregation of renatured products was measured by SEC as follows: supporting column, a series combination of Asahipak GS-520H, GS-620H, GS-720H; column size, 7.6 mm i.d. and 250 mm length each; mobile phase, 0.05M lactic acid (pH 2.5); flow rate, 0.4 mL/min; temperature, 15°C; detection, absorbance at 230 nm.

RESULTS & DISCUSSION

Isolation of α1(I) from shark skin

The denaturation temperature (mid-point, Tm) of shark skin collagen (I) was about 30°C (Fig. 1). A previous report (Piez, 1988) showed that the denaturation temperature of collagen (I) from lower aquatic vertebrates was about 30°C. The denaturation temperature of shark skin collagen is about 12°C lower than that of pig skin collagen (I). The rotation of shark skin collagen (I) sharply decreased at about 27°C, so the renaturation experiment was done below that temperature for shark skin collagen. The specific rotation of native collagen from shark skin was −410.

The denaturation of shark skin collagen to isolate the α1(I) chain was carried out at 40°C. CM-chromatographic patterns of denatured collagen(I) from shark and pig skin were compared (Fig. 2). Chains from shark skin collagen eluted at higher salt concentration than those from pig skin collagen.

Shark skin α1(I) eluted at 0.1M NaCl, while pig skin α1(I) eluted at 0.05M NaCl. SDS-PAGE patterns from the major fractions of CM-chromatography of shark and pig skin collagen (I) were also compared (Fig. 3). The first peak (fractions 7, 8, 9) was identified as α1 with a small shoulder of β11, and the second peak (fractions 10, 11, 12) as β12 as the major component and smaller amounts of γ and larger chains, based on previous work (Kimura et al., 1981). As pointed out by Kimura et al. (1981) in shark skin collagen, the α2 chain does not exist as a monomer, but exclusively as a dimer (β12) combined with α1 chain. The crude α1(I) from the CM-chromatographic fractions was further purified by the repeated use of SEC.

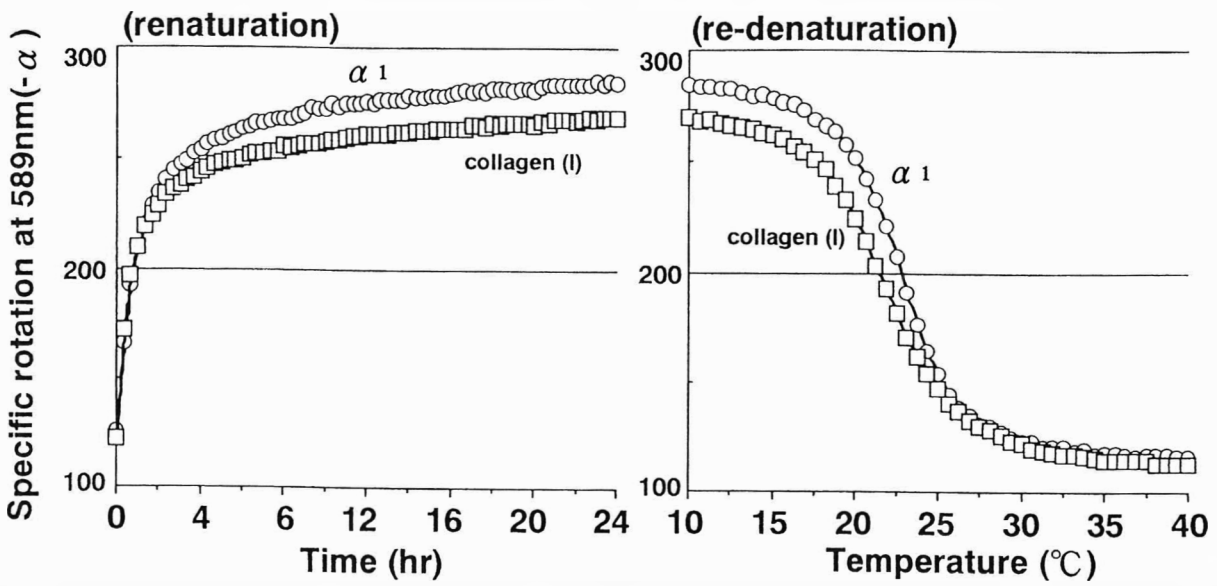

Fig. 4—Renaturation and redenaturation curves of original and α1 chains from shark skin collagen (I) as measured by specific rotation.

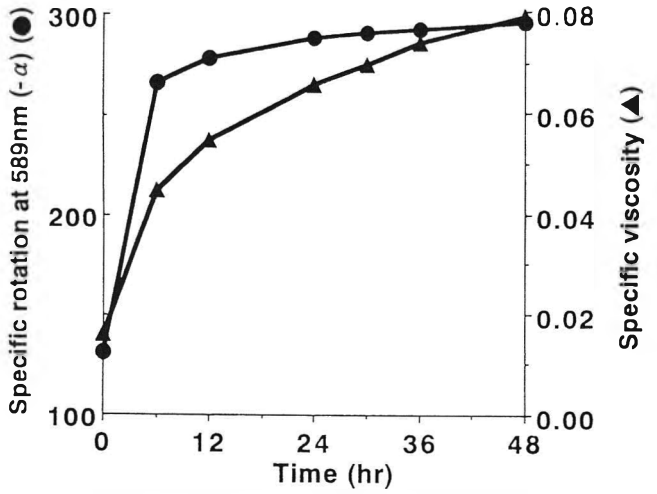

Fig. 5—Renaturation curves of α1 chains from shark skin collagen (I) as measured by specific rotation (●) and specific viscosity (▲).

Renaturation of α1(I) from shark skin collagen

The renaturation process for α1(I) from shark skin was followed using the time-course change of rotation and viscosity. First, rotation changes of α1(I) were examined in four solutions (0:01M sodium phosphate buffer at pH 5, 7, 8 and 0.01M acetic acid at pH 3.6) (data not shown). The greatest specific rotation change was at pH 7.0, while no change was observed in acetic acid. Therefore, the renaturation study of α1(I) was carried out in 0.01M phosphate buffer at pH 7.0 (Fig. 4). Renaturation curves had two steps. For the first 4 hr, the parameter change was rapid and after that, it became slow. After 18 hr, the curve was relatively unchanged. After 48 hr, the specific rotation of α1(I) was constant at −294.7. The renaturation curve reflected two different speeds of reactions. Namely, the first step (renaturation curve of rapid growth) was refolding of random coils to a collagenous helix and the second step was maturation of the helix (Veis, 1964). The constant specific rotation of α1(I) and native collagen (I) at −294.7 and −410, respectively, represents a recovery of rotation of about 72%. It has been known that α chains from mammalian collagen refold with time to partially recover the helical structure of native collagen responsible for the optical rotation increase (Piez and Carrillo, 1964). Therefore, the described renaturation process probably reflected the same conformation change in α1(I) from shark skin collagen as is known for α1(I) from mammalian collagen.

The specific viscosity rose with time, and continued to rise after rotation stopped changing (Fig. 5). This specific viscosity increase corresponded to an intrinsic viscosity increase from 0.26 dL/g (before renaturation, η_0) to 2.77 dL/g (after 24 hr, η_{24}). If the degree of renaturation is expressed as the ratio of η_{24} to the known value of the intrinsic viscosity for native shark skin collagen, 10–15 dL/g, η_n (Kubota and Kimura, 1967), it gives a recovery of about 18–28%. The lag of viscosity change compared with optical rotation change suggests that the interchain association of α1(I) chains to form a collagen-like helical molecule is substantially slower than the intra-chain folding. Similar results have been observed for α1(I) from mammalian collagen (Piez, 1988).

The remelting process for renatured α1(I) was followed as the time-course change of specific rotation (Fig. 4). The renatured α1(I) was denatured by re-heating. This remelting temperature was lower by about 7°C than the denaturation temperature of native collagen. The width of the remelting temperature curve, defined as the temperature range from the beginning of the optical rotation decrease to the end of the rotation change (about 15°C, see Fig. 4), was larger than that for the original native collagen (about 8 °C, see Fig. 1). The greater width of the remelting curve suggested some heterogeneity in the thermal stability of the structure. The results demonstrate the reversibility of α1(I) renaturation and the partial recovery of the collagen-like structure. They show that shark skin collagen (I) has the same ability to unfold and refold into helices as that of higher vertebrates.

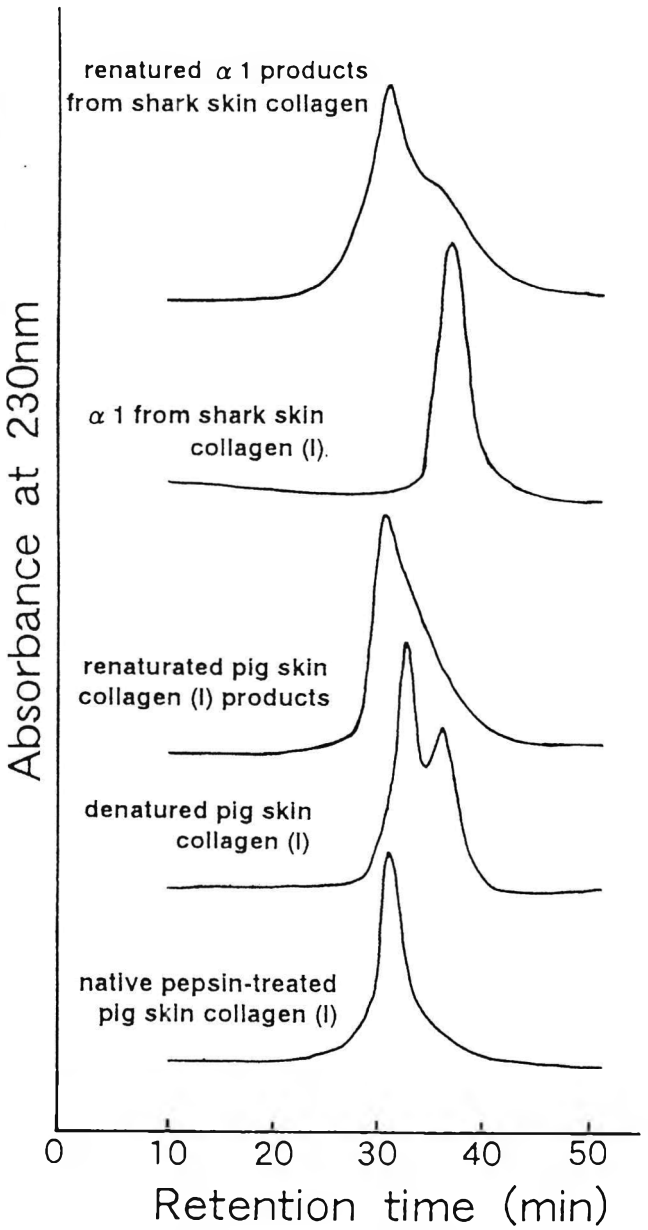

Fig. 6—Elution profile of renatured α1 products from shark skin collagen (I) on SEC-HPLC.

When the rotation increase of α1(I) from shark skin collagen during renaturation was compared with that of denatured collagen (a mixture of α1(I) and other chains), the value of the α1(I) was always larger (Fig. 4). Such a difference has not been reported for chains from calf skin collagen (Veis, 1964). In denatured calf skin collagen, the γ component was easier to renature than the α and β components.

Molecular property of renatured α1(I)

To investigate the molecular size of renatured α1(I), SEC was carried out (Fig. 6). The original native pig skin collagen (I) molecules after treatment with pepsin, which removed the nonhelical region telopeptide, could be separated from the denatured chains (Nakazaki et al., 1994). The telopeptide must be removed because of its strong interaction with the SEC gel. Then pepsin

treated porcine collagen showed one peak (retention time 31 min) while denatured collagen gave broad peaks at 33 min (β chain) and 37 min (α chain). As renaturation progressed, the peaks fused into a somewhat broad peak at about 32 min, near that of the native collagen. Renatured α1 from shark skin collagen (I) gave a native-like peak with a small shoulder at the low-molecular side, showing the formation of a native-like molecular conformation as reported by Tkocz and Kuhn (1969) for α1 and α2 from calf skin collagen by electrophoresis and ultracentrifugation. Piez and Sherman (1970) also reported that α1-CB2 from lathyritic rat skin collagen (I) renatured in the same manner. Our results showed that denatured shark skin collagen (I) and its isolated α1 also refolded reversibly to obtain the native-like collagen structure through renaturation.

Overall, the results imply that the structure of shark skin collagen (I) was basically similar to that of higher land vertebrate collagens (I), although there was an apparent difference in imino acid residue contents that lowered the denaturation temperature (Kimura et al., 1981). Taking into account the reports on fish gelatins by Berg et al. (1985) and Leuenberger (1991), we concluded that shark collagen or gelatin could possibly be used in applications where a high solution viscosity with low gel strength is desired. For example, with food, such a property may be useful in the manufacture of ice cream and as coating agents for frozen fresh fish.

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Ms received 9/19/94; revised 2/18/95; accepted 5/15/95.

Thermal Stability and Gel-Forming Ability of Shark Muscle as Related to Ionic Strength

HUI-HUANG CHEN

ABSTRACT

Thermal stability and gel-forming ability of silvertip, hammer-head and thresher shark as affected by ionic strength were investigated. Low temperature endothermic peaks (LTEP, between 30 and 44°C) were observed in differential scanning calorimetric (DSC) thermograms of the shark muscles and surimi samples. Increases in ionic strength reduced the thermal stability of surimi as shown by depression of transition temperature, denaturation enthalpies and LTEP. Although gel strength of heat-treated surimi increased with increasing NaCl concentration, the three species had different sensitivities to ionic strength. The highest NaCl-induced gel-forming ability of surimi was achieved at 0.5M salt concentration for silvertip and thresher; at 1.0M for hammer-head shark.

Key Words: shark, ionic strength, thermal stability, DSC, gel-forming

INTRODUCTION

THE GELATION CHARACTERISTICS of surimi are influenced by ionic strength. Steps assumed to occur to form the "sol" when fish meat is minced with salt and allowed to set are: (1) solubilization of salt soluble proteins; (2) dissociation of myosin filaments into individual molecules; (3) partial unfolding of α-helix region of myosin heavy chain; (4) formation of an ordered gel network by aggregation through hydrophobic and electrostatic interactions (Niwa and Miyake, 1971; Liu et al., 1982; Roussel and Cheftel, 1990). Myosin is the most important constituent during heat gelation of meat, and its active sites (such as LMM, S1 and S2) are swollen and unfolded after setting. A reversible filamental structure would reconstitute and form a stronger network through intrafilamental cross-linking of LMM (Egelandsdal et al.,1986). Sano et al. (1988) suggested that there are two major stages of myosin gelation with dynamic heating. The first stage occurs in the 30-41°C range and the second in the 51-80°C range. The first stage mainly depends on interactions among the tail portions. The second stage is attributable to interactions among the head portions of myosin molecules (Sano et al., 1990). However, the actin and regulatory proteins have an important influence on the gel-forming ability of myosin in the presence of NaCl (Samejima et al., 1981).

Sharks are the major source of raw materials for manufacturing surimi products in Taiwan. Few studies have been reported on the gel-forming ability of shark muscle and its dynamic thermal stability (Shimizu et al., 1981; Koreeda, 1982; Jeng and Hwang, 1978; Chow, 1984). Many different chemical techniques have been applied to study the properties and behavior of fish meat protein during heating. However, classical methods to measure protein denaturation provide only partial information on this phenomenon, and the extraction and purification process might affect the native state of the proteins. Differential scanning calorimetry (DSC) provides a direct and simple method to study the thermal transitions of muscle protein without mechanical force or chemical destruction (Samejima et al., 1983; Beas et al., 1990). Our objective was to use DSC to study the thermal transition of shark muscle proteins and surimi with different NaCl concentrations. Also, the physical properties of heat-treated surimi were determined to evaluate effects of ionic strength.

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MATERIALS & METHODS

Materials

Silvertip (*Carcharhinus brachyurus*), hammer-head (*Sphyrna lewini*) and thresher sharks (*Alopias pelagicus*) were caught by commercial vessel off the North-east coast of Taiwan. The fish were held in ice and brought to the laboratory within 24 hr after being caught. The sizes and pH of the six experimental fish were compared (Table 1).

Preparation of muscle proteins and surimi

Surimi. The dorsal muscle was dissected into small pieces in such way as to avoid extraction of connective tissue. This dissected dorsal muscle ("whole muscle") from each species was mixed together and minced to cubes of less than 3 mm. After washing three times with cold water (water : flesh ratio, 3:1), the samples were centrifuged for 2 min. The water content of the washed and dewatered minces was determined with a quick water content analyzer (Mettler LP16C) and adjusted to 78%. NaCl at concentrations of 1.0, 2.5, 3.5, 5.0 or 8.0g was added to 100g minced muscle which was then ground for 4 min in an ice bath giving final salt concentrations of 0.2, 0.5, 0.7, 1.0 and 1.6M.

Heat-treated surimi. The surimi was injected into 10 cm circumference, 15 cm length polyvinylidene chloride casing and heated at 85°C for 30 min ("surimi sausage"). The surimi sausage was then cooled with cold water immediately after heating and kept at 4°C overnight before physical properties were measured.

Sarcoplasmic proteins. Minced muscle was immersed in a 0.1M KCl, 2 mM $MgCl_2$, 20 mM KH_2PO_4 solution (pH 7, μ=0.1) for 45 min. The supernatant of the slurry after centrifugation was concentrated and used for DSC analysis (Hastings et al., 1985).

Leached muscle. Minced muscle was leached three times with distilled water (water:flesh ratio, 10:1) and stirred for 5 min at 4°C. The leached muscle was dewatered with filter paper.

Muscle depleted of sarcoplasmic proteins. Minced muscle was placed in a 0.1M KCl solution (pH 6) and stirred for 4 hr at 4°C to remove the sarcoplasmic proteins (Beas et al., 1990, 1991).

Collagen. Connective tissue was dissected from the myocommata of the sharks as a source of collagen.

Thermal analysis

Differential scanning calorimetry (DSC) tests were performed with a DuPont TA 2000 system. The samples (about 15 mg) were placed in hermetic pans and scanned at 10°C/min over the range 10–100°C. A pan with 12 mg distilled water was used as reference. Total denaturation enthalpies (ΔH) were determined by measuring the area between DSC

Table 1—Dimensions of sharks

	Weight (kg)	Length (cm)	pH[a]
Silvertip shark	10.8	109	5.88 ± 0.02[b]
	13.1	122	5.90 ± 0.04
Hammer-head shark	26.4	188	6.01 ± 0.01
	20.5	163	5.95 ± 0.03
Thresher shark	15.4	132	5.82 ± 0.03
	12.0	108	5.85 ± 0.04

[a] The filtrate of homogenized solution of 5g dorsal muscle and 45 mL deionized water was determined with a pH meter.

[b] All pH values of dorsal muscle are means of three determinations ± standard deviation.

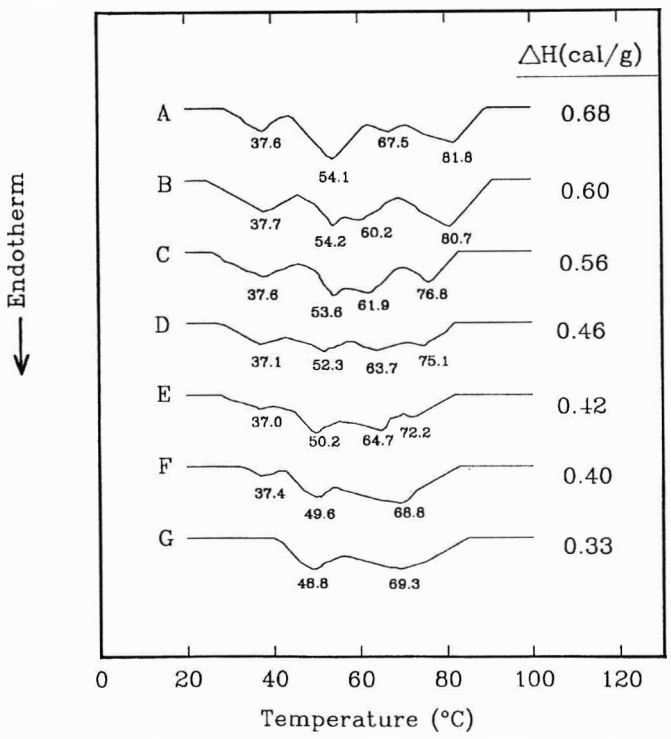

Fig. 1—DSC thermograms of silvertip shark muscle and surimi showing effects of salt concentration. (A) whole muscle; (B), (C), (D), (E), (F), and (G) were surimi with 0, 0.2, 0.5, 0.7, 1.0, and 1.6M NaCl.

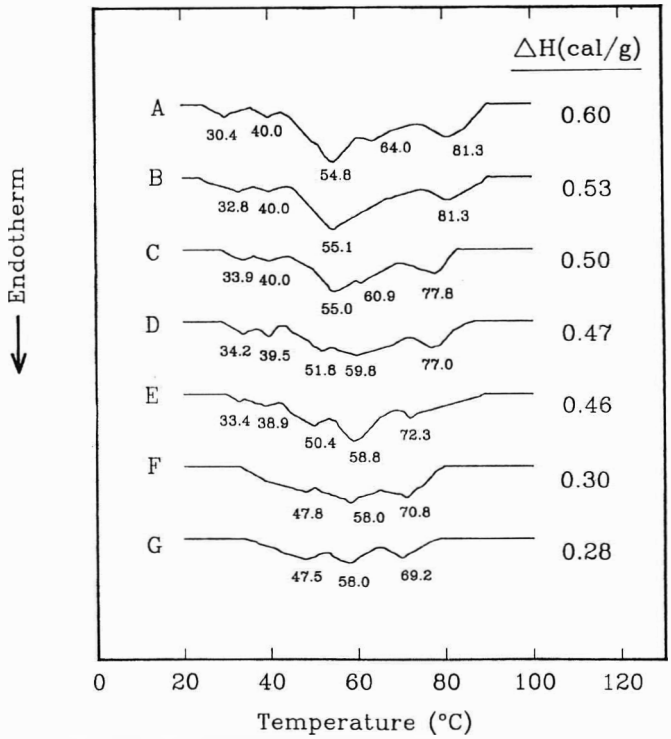

Fig. 2—DSC thermograms of hammer-head shark muscle and surimi showing effects of salt concentration. (A) whole muscle; (B), (C), (D), (E), (F), and (G) were surimi with 0, 0.2, 0.5, 0.7, 1.0, and 1.6M NaCl.

transition curve and the base line. The temperature and enthalpy calibrations were performed with indium and benzoic acid to calibrate the melting point. Four replicate scans were made for the whole muscle of each shark species, and three replicates for the surimi and muscle proteins. The temperature at each peak maximum was recorded as the transition temperature (T_m).

Physical properties of surimi products

Gel strength. Each surimi sausage was cut into 30 mm sections. The gel strength was determined with a rheometer (Sun Rheometer, CR-150) using a 5mm diameter spherical head plunger to press into one end of each sample with 60 mm/min deformation rate. The breaking force was the force required to break a sample. The gel strength was calculated as breaking force (g) times deformation (cm), and the mean value of six replicates at each NaCl concentration was determined.

Expressible water content. The surimi products were cut into 3mm sections, folded in filter paper and expressed with 10kg/cm² loading for 20 sec. The expressible water content (%) was determined as the weight loss divided by initial weight of sample. At each NaCl concentration, six replicates were measured.

NaCl-induced gel-forming ability. The inverse of salt concenteration was taken to represent the salt concentration efficiency (C). The intensity in gel strength increase (G) was defined as the difference in gel strength between a given sample and surimi sausage without NaCl. The mean value of six replicates of the NaCl-induced gel-forming ability of surimi at each salt concentration was determined as CG (g·cm/M).

Statistical analysis

Statistical analysis of the physical properties data of surimi sausage was performed using the system developed by SAS Institute, Inc. (1985). When analysis of variance (ANOVA) revealed a significant effect ($P<0.05$), data means were compared with the least significant difference (LSD) test.

RESULTS & DISCUSSION

Thermal analysis of shark muscle and proteins

DSC thermograms of shark muscle showed a characteristic profile (Fig. 1A, 2A and 3A). Silvertip shark muscle showed a small endothermic peak (T_m=37.6°C), but hammer-head and thresher shark muscle showed two peaks within 30–44°C. These small peaks observed at relatively low temperature were defined as low temperature endothermic peaks (LTEP).

For further determination of LTEP, the muscle proteins which were isolated from silvertip shark were investigated. The sarcoplasmic protein showed peaks at 44.8, 54.5, 63.1, 68.5 and 76.0°C (Fig. 4A). The thermograms of leached muscle and muscle depleted of sarcoplasmic proteins had LTEP at 39.2 and 37.0°C, respectively (Fig. 4B, C). On the thermograms of muscle depleted of sarcoplasmic proteins fraction, a clear endothermic peak (T_m=60.1°C) was observed (Fig. 4C). The surimi from that fraction also showed a transition peak around 61°C (Fig. 4E, F). The peak at T_m=53.1°C in Fig. 4C did not appear in Fig. 4E. Peaks around 53 and 61°C in the shark DSC profile were attributed to myosin and actomyosin complex denaturation, respectively (Chen and Chen, 1994). The shrinkage or disappearance of the heat denaturation peak around 53°C suggested that in the shark muscle that had been ground with 0.5M NaCl or stirred with 0.1M KCl, the interaction of myosin and actin was accelerated and the amount of free myosin was reduced.

The LTEP occurred in DSC thermograms of leached muscle and muscle depleted of sarcoplasmic proteins but not in sarcoplasmic protein (Fig. 4A, B, C). Thus, the LTEP of sharks were considered to be caused by the heat denaturation of myofibrillar proteins. Hastings et al. (1985) suggested that the LTEP of cod proteins around 35°C might correspond to the first transition of myosin and collagen. However, the LTEP of sharks cannot be attributed to collagen transition because collagen's T_m was observed at 55.8°C and formed a large single peak. The actin transition temperature of sharks is around 81°C (Chen and Chen, 1994). We concluded that the LTEP of shark muscle was due to the denaturation of myosin, which occurs at a lower temperature. The first transition peak in DSC thermograms of some muscle proteins such as marlin (Lo et al., 1991), carp (Akahane et al., 1985) and rabbit (Wright et al., 1977; Wright and Wilding, 1984), is distributed over the range 36–47°C and corresponds to

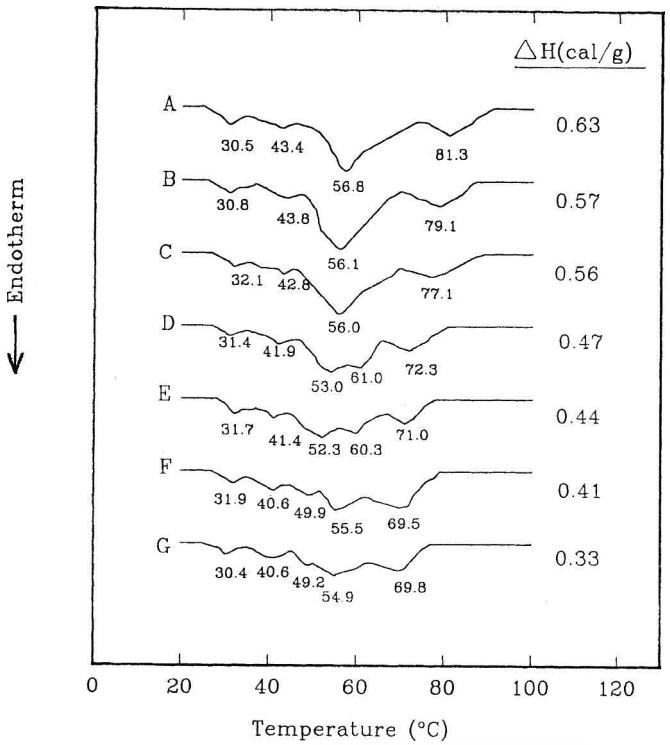

Fig. 3—DSC thermograms of thresher shark muscle and surimi showing effects of salt concentration. (A) whole muscle; (B), (C), (D), (E), (F), and (G) were surimi with 0, 0.2, 0.5, 0.7, 1.0, and 1.6M NaCl.

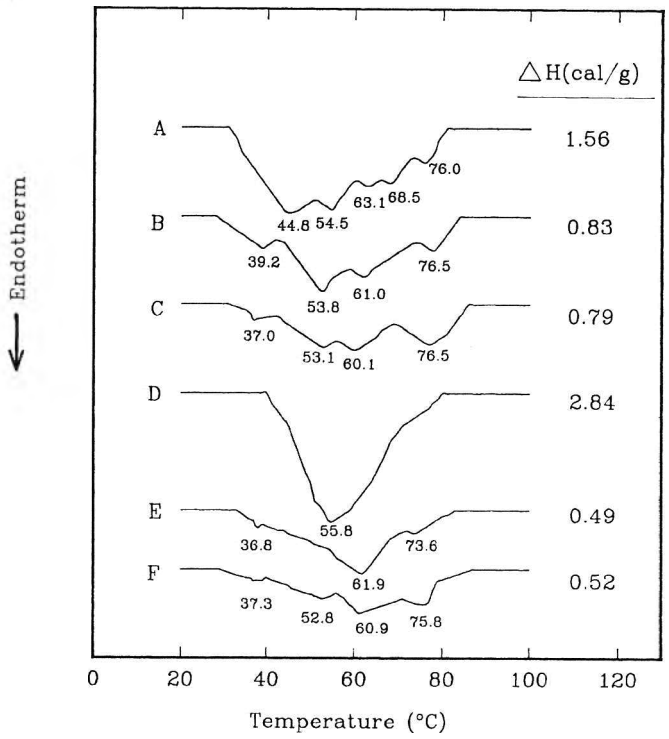

Fig. 4—DSC thermograms of silvertip shark muscle protein and surimi. (A) Sarcoplasmic protein; (B) leached muscle; (C) muscle depleted of sarcoplasmic protein; (D) collagen; (E) surimi of muscle depleted of sarcoplasmic protein (0.5M NaCl addition); (F) surimi of muscle depleted of sarcoplasmic protein (no NaCl addition).

the transition temperatures of their respective myosin tail. Similarly, the LTEP of shark muscle might be caused by heat denaturation of its myosin tail which is developed by the destruction of the sarcolemma of shark muscle.

Thermal analysis of shark surimi

No significant difference occurred in DSC thermograms between shark muscles and the surimi without added salt. Evidently, mechanical force of the grinding process did not affect heat stability of protein. However, as NaCl concentration increased, the T_m of myosin and actin transition both shifted to lower temperatures and the peak areas were also reduced. Those LTEP became gradually less obvious as NaCl concentration increased. The LTEP did not occur at 1.0M and 1.6M salt concentration for hammer-head and silvertip shark surimi (Fig. 2F and 1G). Thus, increasing ionic strength decreased the thermal stability of surimi; T_m, ΔH and LTEP were lowered. This indicated that the protein structure had been destabilized.

As NaCl concentration of surimi increased, the endothermic peak area of actomyosin complex increased. The actin transition peak seemed to merge with the actomyosin complex transition peak when the NaCl concentration of silvertip shark surimi reached 1.0M (Fig. 1F). We postulated that the increase of ionic strength solubilized myofibrillar protein and induced free myosin associated with actin to form actomyosin. The lack of the actin transition peak after addition of 1.0M NaCl in silvertip shark surimi might be due to inadequate instrument sensitivity.

Physical properties of surimi sausage

The breaking force of surimi sausage increased with increasing NaCl concentration. Deformation increased at lower NaCl concentration and then decreased when NaCl concentration reached 1.6M (Table 2). The gel strength reached the maximum at 0.5M NaCl concentration and then leveled off for silvertip shark. For hammer-head shark, maximum gel strength was found at 1.0M NaCl. Thresher shark surimi sausage showed poor gel-forming ability without added NaCl. Probably because of inadequate sensitivity of the instrument, there was no apparent pressure difference in deformation during compression and the gel strength was unmeasurable (Table 2).

With increasing NaCl concentration, the water content of the shark surimi sausage increased at lower NaCl concentration and then decreased at above 1.0M (Table 2). Although the shark surimi sausage without NaCl addition or with 1.6M NaCl con-

Table 2—Physical properties of heated shark surimi (surimi sausage) as related to salt concentration

Salt conc of surimi (M)	Breaking force (g)	Deformation (cm)	Gel strength[g] (g-cm)	Water content (%)	Expressible water content (%)
			Silvertip shark		
0	502^{d}	0.69^{d}	348^{c}	74.3^{d}	50.6^{a}
0.2	752^{c}	0.81^{bc}	609^{b}	75.9^{c}	41.7^{b}
0.5	961^{b}	1.09^{a}	1033^{a}	76.5^{b}	33.2^{c}
0.7	975^{b}	1.09^{a}	1085^{a}	77.0^{a}	30.5^{c}
1.0	985^{b}	1.05^{a}	1034^{a}	76.8^{ab}	29.6^{c}
1.6	1217^{a}	0.88^{b}	1071^{a}	74.1^{d}	40.8^{b}
			Hammer-head shark		
0	393^{e}	0.79^{d}	310^{e}	74.0^{e}	53.2^{a}
0.2	407^{e}	0.84^{cd}	342^{e}	75.5^{cd}	44.1^{b}
0.5	647^{d}	0.93^{b}	602^{d}	76.3^{ab}	34.5^{c}
0.7	770^{c}	0.99^{ab}	762^{c}	76.8^{a}	33.5^{c}
1.0	927^{b}	1.10^{a}	1020^{a}	75.3^{d}	31.9^{c}
1.6	1021^{a}	0.96^{b}	986^{ab}	73.6^{f}	37.6^{bc}
			Thresher shark		
0	180^{e}	—[h]	—	74.2^{d}	73.9^{a}
0.2	307^{d}	0.64^{d}	196^{e}	75.9^{ab}	62.8^{b}
0.5	568^{c}	0.96^{a}	540^{d}	76.4^{a}	55.1^{bc}
0.7	640^{bc}	0.95^{a}	608^{bc}	76.5^{a}	49.1^{c}
1.0	705^{b}	0.90^{ab}	635^{b}	76.2^{a}	43.0^{d}
1.6	836^{a}	0.84^{bc}	702^{a}	75.1^{c}	41.9^{d}

[a-f] For each column within a shark species, different superscripts signify a significant difference ($P < 0.05$). (Values shown are means of six replicates)

[g] Gel strength = breaking force × deformation.

[h] There is no apparent pressure difference in deformation during compression.

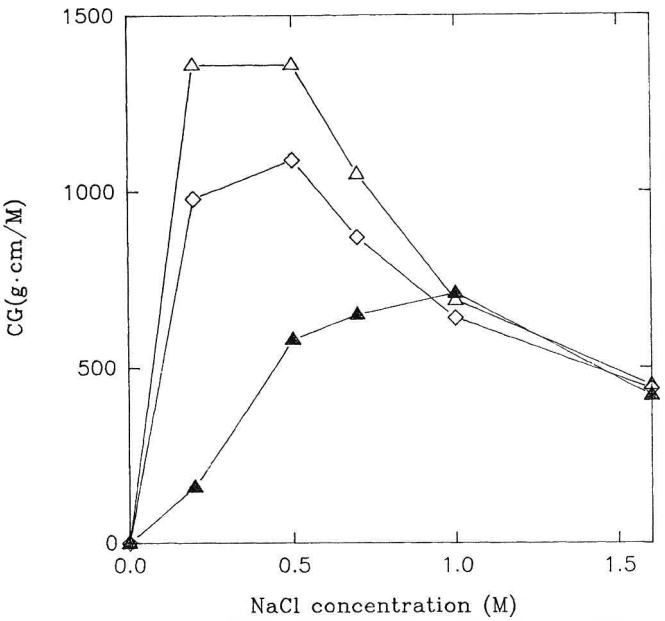

Fig. 5—NaCl-induced gel-forming ability (CG) of sharks, where C is the inverse of salt concentration and G is the difference of gel strength between the sample and surimi sausage without NaCl. △: silvertip shark; ▲: hammer-head shark; ◇: thresher shark.

centration had lower water content, the surimi sausage without added salt had a lower breaking force and deformation indicating a soft, crumbling texture. The surimi sausage with 1.6M NaCl had high breaking force and low deformation indicating a hard, solid texture. Meanwhile, the expressible water content of thresher shark surimi sausage decreased with increasing NaCl concentration. The expressible water content of silvertip and hammer-head shark decreased to the lowest value at 1.0M and then increased at 1.6M NaCl (Table 2). The increase of water content and deformation and the decrease of expressible water content with increasing salt concentration might be due to salt solubilizing myofibrillar proteins and thus increasing water hydration capacity (Niwa, 1992). However, high ionic strength would diminish the positive effect of F-actin on gel strength since electrostatic forces would be weakened (Asghar et al., 1987). Also, the salting-out denaturation of protein occurred at high salt concentration (Takahashi et al., 1993). Thus, the shark surimi sausage with high ionic strength had low deformation, low water content but high expressible water content.

The addition of NaCl to surimi would solubilize myofibrillar proteins and accelerate the formation of protein networks. However, a given ionic strength resulted in a different degree of gel-forming ability in different shark. The NaCl-induced gel-forming ability (CG) of surimi increased rapidly up to 0.5M NaCl concentration in silvertip and thresher shark surimi but decreased thereafter (Fig. 5). Silvertip and thresher shark surimi showed high NaCl-induced gel-forming ability at lower ionic strength. Thus, muscle from these two sharks was more sensitive to ionic strength. The highest CG value was reached at 1.0M NaCl for hammer-head surimi (Fig. 5), which indicated that the muscle required higher ionic strength to maximize the gel-forming ability of surimi.

Ionic strength and gel-forming mechanism

Texture results from heating of fish protein and salt might be associated with destabilization effects of salt on proteins. The high ionic strength solubilized the myosin in monomeric units, and interactions among the tail portions and/or between the tail and head portions would occur. During heating in DSC analysis, changes in conformation of myosin molecules in shark surimi, including solubilization and denaturation of myosin tails, might occur at a low temperature (30–43°C), corresponding to the LTEP. At higher temperatures (47–57°C), the head portions of the myosin molecule might project from the filament which would enable interactions among head portions. Further interactions between myosin and actin could then form the actomyosin complex (at 54-67°C).

Sano et al. (1988) reported that myosin was essential for the development of gel strength in surimi products and that the thermal gelation of myosin had two major stages at 31–41°C and 51–80°C. These temperature ranges agreed fairly well with that of the LTEP and actomyosin complex transition peak of shark surimi. The shrinkage of LTEP was observed and accompanied with the increase of peak area of actomyosin complex denaturation as well as increase of gel strength of shark surimi sausage. Results suggest a relationship between these two transition peaks and the gel-forming ability of shark surimi with changes of ionic strength.

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Ms received 11/4/94; revised 5/4/95; accepted 6/9/95.

Functional Properties and Shelf Life of Fresh Surimi from Pacific Whiting

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ABSTRACT

Total aerobic plate count (APC), shear stress, shear strain, and color of fresh Pacific whiting surimi stored at 5°C were determined at day 0, 1, 3, 5, and 7. Frozen surimi was prepared with four levels of cryoprotectants (0, 3, 6, and 9%) and was compared with fresh surimi for gel-forming ability. Fresh Pacific whiting surimi had a shelf life of 5 days. The gel functionality remained unchanged throughout the storage time. Strain values of fresh surimi were not different from those of frozen surimi with 9% cryoprotectants, but stress values of fresh surimi were almost three times higher than those of frozen surimi.

Key Words: fresh surimi, shelf life, Pacific whiting

INTRODUCTION

PACIFIC WHITING (*Merluccius productus*) is the most abundant species off the coast of Washington, Oregon, and Northern California with a total allowable catch of 140,000 to 250,000 metric tons a year (NMFS, 1993). Processing of surimi from Pacific whiting was not commercialized in the U.S. until 1991 for at-sea operations and 1992 for shore-side operations. Surimi is stabilized fish myofibrillar proteins. It is produced by continuous processing steps of heading, deboning, mincing, washing, dewatering, and freezing with cryoprotectants. The washing and dewatering processes free fish meat from sarcoplasmic proteins, fat, and undesirable materials such as skin and fine bones. High-quality surimi is naturally odorless and colorless.

All commercial surimi is kept frozen. Cryoprotectants are required to minimize protein denaturation during frozen storage. Sucrose (4%) and sorbitol (4–5%) are commonly used along with 0.3% sodium phosphate. Several studies of different cryoprotectants to measure changes of protein functionality during frozen storage have been conducted (Arai et al., 1970; Noguchi et al., 1975, 1976; Park et al., 1987a, b; Park and Lanier, 1990; Sych et al., 1990a, b). A higher level of cryoprotectants contributes a notable degree of sweetness to surimi-based foods. Consumers often consider high sugar or sweeter products as undesirable (Baute, 1994). Search for non-sweet or low-sweet carbohydrates as effective cryoprotectants has been reported by Arai et al. (1984), Watanabe et al. (1985), Sase et al. (1987), Park et al. (1988), Sych et al. (1990a, b), and Lanier (1993). Polyalcohols, polydextrose, maltodextrin, and starch hydrolysates have been investigated. Though their cryoprotective effects were comparable to sucrose or sorbitol, these alternatives have not been used due to legal issues and the lack of commercial tests.

During frozen storage, several changes occur in fish muscle proteins. These include denaturation, ice crystallization, dehydration, and changes in intramolecular conformation, such as salt-soluble protein, pH, ionic strength (Park, 1994b). Many proteins have exhibited instability as measured by the partial loss of functionality at subfreezing temperatures. The deterioration of proteins during frozen storage is reflected by their sharp decrease in gel-forming ability, water-holding capacity, and fat-emulsifying capacity (Iwata and Okada, 1971; Cheng et al., 1979; Holmquist et al., 1984; LeBlanc et al., 1988; Park et al., 1988; Hsu, 1990; Yoon and Lee, 1990; Sych et al., 1991). Quality changes due to freeze-denaturation can be minimized during storage with cryoprotectants. However, losses of salt-soluble proteins and gel-forming ability still occurred in cryoprotected frozen surimi. Park et al. (1988) and Sych et al. (1991) reported a loss of salt-soluble proteins in cryoprotected surimi during frozen storage. Furthermore, gel functionalities, stress and strain, of surimi mixed with cryoprotectants decreased notably due to freezing (Hsu, 1990; Sych et al., 1991; Simpson et al., 1994) and continued to decrease over prolonged storage (Park et al., 1988; Sych et al.. 1990b).

Before 1960, all surimi used in the Japanese kamaboko industry was fresh surimi (Okada, 1990). There was no method available to control freeze-denaturation until Nishiya developed a technique by which freeze denaturation of proteins in Alaska pollock surimi could be prevented (Matsumoto, 1979). They added cryoprotectants such as sucrose and sorbitol to stabilize myofibrillar proteins. The use of fresh surimi in the U.S. was almost impossible due to the geographical distance of Alaska pollock surimi plants (Dutch Harbor and Kodiak, AK) until shore-side operations were established in Oregon. Benefits of using fresh surimi over frozen surimi are: fresh surimi can be produced at a lower cost without additives and freezing process; it can be used for no-sugar or low-sugar products; it can add value to some products like 'never-frozen' surimi-based crabmeat; it can show better gel functionality and be used further at a reduced level.

The objectives of our study were to investigate gel functionality and shelf life of fresh Pacific whiting surimi and compare it with currently available frozen surimi.

MATERIALS & METHODS

FRESH PACIFIC WHITING SURIMI was collected from Point Adams Packing Co. (Hammond, OR) immediately after the dewatering process and before mixing with cryoprotectants and other ingredients. Fresh surimi was placed in a polyethylene bag, kept in ice, and transported to the OSU Seafood Laboratory within 30 min. The moisture content of fresh surimi was $81 \pm 0.7\%$ by a rapid microwave method (Morrissey et al., 1993). The testing consisted of three parts: (1) shelf life and functional quality of fresh surimi; (2) functional quality of frozen surimi at different levels of cryoprotectants during storage; and (3) comparison of fresh surimi with frozen surimi.

Fresh surimi

Fresh Pacific whiting surimi was packed in food-grade plastic bags, 1.00 ± 0.05 kg each. All samples were sealed without vacuum and kept at 3–5°C. Fresh surimi samples were evaluated for total aerobic plate count (APC), shear stress, shear strain, and color at day 0, 1, 3, 5, and 7 during storage. All experiments were repeated twice.

Frozen surimi

Frozen surimi was prepared by mixing fresh surimi with 0, 3, 6, and 9% cryoprotectants, a mixture (1:1) of sucrose and sorbitol (ICI Specialties, New Castle, DE), and 1% beef plasma protein (BPP; AMPC, Inc., Ames, IA) based on total weight. Mixing was conducted for 6 min in a cold room (5°C) using a Hobart mixer (model S301, Hobart Mfg Co., Troy, OH). Sub-samples of 1.00 ± 0.05 kg were packed in food-grade bags, sealed without vacuum, frozen in a blast freezer (−27°C),

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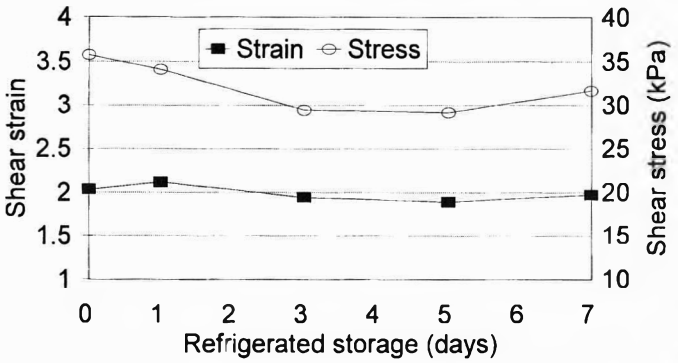

Fig. 1—Gel functionality (shear strain and shear stress) of fresh Pacific whiting surimi observed as related to storage time.

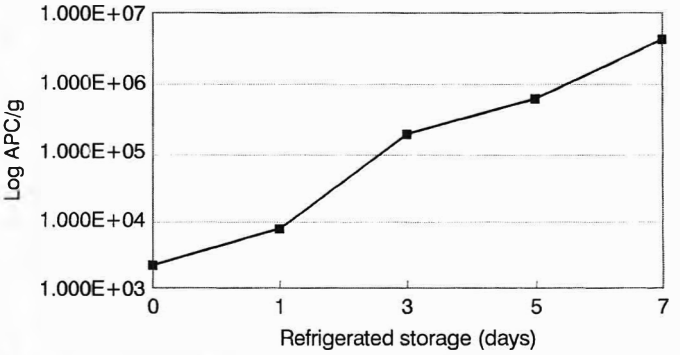

Fig. 2—Total aerobic plate count (APC) of fresh surimi during refrigerated storage.

and kept in the freezer at −18°C. Frozen surimi samples were evaluated for textural properties and color before freezing (day 0), and after 1 day, 2 wk, and 2 mo of frozen storage. Each treatment, with different levels of cryoprotectants, was prepared separately from different batches of fresh surimi. All experiments were repeated twice.

Freezing effects on surimi with different levels of cryoprotectants

Fresh Pacific whiting surimi was mixed with 0, 3, 6, and 9% cryoprotectants, a mixture (1:1) of sucrose and sorbitol, and 1% BPP (w/w) for 6 min in a refrigerated room. We studied changes of shear stress and shear stain of surimi gels due to freezing. All treatments were prepared from the same batch of fresh surimi. Experiments were conducted before freezing and after 20–24 hr of frozen storage. The experiment was repeated four times.

Total aerobic plate count

A 10-g surimi sample was homogenized for 2 min with 90 mL of 1% sterile bacto-peptone water (Bacto-Peptone; Difco Laboratories, Detroit, MI). Higher dilutions were made, poured and spread on Petrifilms (3M, St. Paul, MN) for aerobic plate count. Petrifilm samples were incubated at 37°C for 48 ± 3 hr. The total number of colonies were counted and reported as APC/g sample.

Gel preparation

Moisture content of each sample was determined before gel preparation using a microwave method described by Morrissey et al. (1993). All gel formulations (with salt and BPP) were adjusted to equal moisture at 80.8–80.9%, using ice if necessary. Protein content was calculated based on moisture, cryoprotectants, and surimi content in each gel formulation. Surimi samples were comminuted with 2% salt, 1% BPP (based on total weight), and ice water in a Stephan vertical vacuum cutter (model UM 5 Universal; Stephan Machinery Corp., Columbus, OH). Gels were prepared as described by Park et al. (1994) and cooked immediately at 90°C for 15 min. Gels removed from the water bath were chilled in ice water and stored overnight in a refrigerator.

Shear stress and shear strain by torsion method

Gel samples were removed from the refrigerator and kept at room temperature (~23°C) for 2 hr prior to the torsion test. Gels were cut into 2.9 cm length and both ends glued to plastic discs with glue (Krazy Glue Inc., Itasa, IL). Twelve sample specimens were used for each test. All gels were milled into an hourglass shape with a minimum diameter of 1.0 cm in the center. Then, each gel was placed in a Torsion Gelometer (Gel Consultant, Raleigh, NC). By twisting samples, shear stress and shear strain were calculated by the methods of Hamann (1983) and recorded on a strip chart. Shear stress indicates the strength and hardness, while shear strain denotes the cohesive nature.

Color test

A Minolta Chroma Meter CR-300 (Minolta Camera Co. Ltd., Osaka, Japan) was used to measure the color of gels (five subsamples each). CIE L*a*b* values were measured, where L* represented lightness, a* for greenness, and b* for blueness. Whiteness was calculated as whiteness index, L*-3b*, as suggested by Park (1994a).

Statistical analysis

Analysis of variance (STATGRAPHICS, 1992) was conducted on torsion and color data to determine the significance of treatments. Least significant difference (LSD) at $p \leq 0.05$ was used to determine significant differences between mean values.

RESULTS & DISCUSSION

Shelf life and gel functionality of fresh surimi

Shear stress and shear strain of gels made from fresh Pacific whiting surimi during storage (3–5°C) for 7 days were compared (Fig. 1). During storage, the stress slightly decreased but strain value remained virtually unchanged. Stress values were between ~29 and ~36 kPa and strain values were 1.89 to 2.12. In general, strain values of gels were somewhat low. Hamann et al. (1990) suggested that a good commercial product could be made from surimi with a strain value >2.0. The slightly low strain value we observed was probably due to high moisture content of the finished gels (80.8–80.9%) compared with normal gels (78% moisture). However, Morrissey et al. (1992) suggested that the lower limit of strain of surimi gel to make a quality product was 1.9 which correlates with the traditional Japanese double-fold test for surimi.

Total aerobic plate count (APC) increased logarithmically with continued storage days (Fig. 2). APC was 10^3 at day 0, 10^5 at day 3 and day 5, and exceeded 10^6 at day 7. Microbial quantity of fish mince $\leq 10^6$ APC/g is acceptable according to Dymsza et al. (1990). They recommended that the quality cut-off for fish mince be 10^6 APC/g. Day 5 appeared to be the last day of storage before fresh surimi exceeded 10^6 APC/g. Also, at day 7, fresh surimi exhibited a mushy flesh and strong undesirable smell. This confirmed studies by Lin et al. (1993) which showed that levels of TMA and DMA in fresh surimi did not increase until day 7.

Both L* values and +b* values of fresh surimi did not change for the first 5 days of refrigerated storage, but decreased ($P<0.05$) at day 7. This was a result of protein decomposition and denaturation that led to the loss of translucency of fish meat (Ledward, 1992). However, a* values did not change throughout storage. As a result, whiteness (L*-3b*) of gels made from fresh Pacific whiting surimi during day 0 to day 5 of storage, (64.77–65.57), did not change (Table 1). After 7 days storage, however, it decreased to 61.59 ($P<0.05$).

Frozen surimi at different levels of cryoprotectants

Shear stress and shear strain of surimi gels without cryoprotectants markedly decreased after 1 day freezing (Fig. 3a and b). This was due to freeze-denaturation which caused changes in

Table 1—Effects of storage time on gel color of fresh surimi

Day	L*	a*	b*	Whiteness
0	82.15[a]	−2.40[a]	5.65[a]	65.19[a]
1	82.39[a]	−2.48[a]	5.61[a]	65.57[a]
3	82.15[a]	−2.29[a]	5.55[a]	65.51[a]
5	81.49[ab]	−2.34[a]	5.58[a]	64.77[a]
7	80.46[b]	−2.42[a]	6.29[b]	61.59[b]

[ab] Means in the same column followed by different superscripts are significantly (P<0.05) different within the same color parameter.

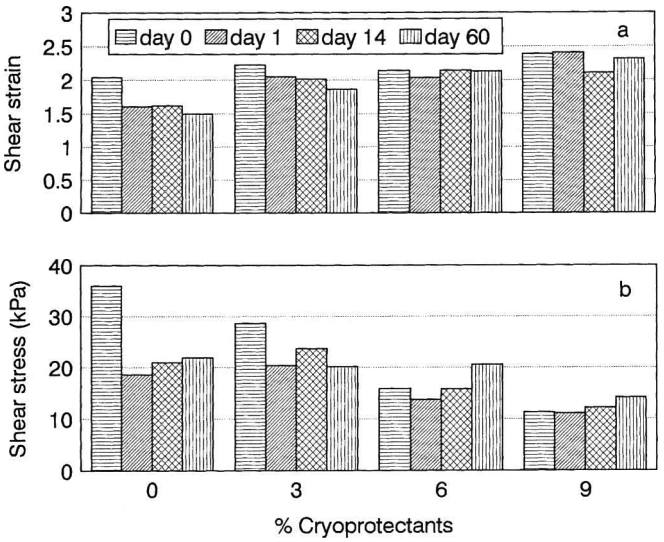

Fig. 3—Shear strain and shear stress of frozen surimi with different levels of cryoprotectants measured as related to storage time.

functional properties (Sikorski et al., 1976) and ice crystal growth which substantially damaged fish tissue (Lawrence et al. 1986). Texture deterioration caused by protein denaturation, upon freezing, appeared to be minimized as levels of cryoprotectants increased (Fig. 3a and b): stress values decreased 48.2%, 28.8%, 13.37%, and 0% in samples with 0%, 3%, 6%, and 9% cryoprotectants, respectively.

During frozen storage, shear strains of surimi with 0% and 3% cryoprotectants markedly decreased (21.8% and 7.9%, respectively) and continued to decrease as storage time increased. However, shear strains of surimi with 6% and 9% cryoprotectants decreased only slightly with storage time. This confirmed a previous study by Yoon and Lee (1990) that reported higher levels of cryoprotectants produced more cohesive gels due to the better protected proteins in frozen surimi. Shear stress values of surimi gels with higher levels of cryoprotectants also did not decrease during frozen storage. However, shear stress seemed to increase slightly with extended frozen storage (day 14 and 60).

Unexpectedly, we observed a slight rising trend of shear strain values of gels made from fresh surimi (day 0) as level of cryoprotectants increased ($P<0.05$) (Fig. 3a). A decreasing trend was observed in shear stress values (Fig. 3b). The rising trend of shear strain values was probably due to a quality difference in each batch of fresh surimi used. To verify this, two additional batches of fresh surimi were evaluated.

All surimi samples showed a slight change in L* values and a* values upon freezing and remained stable during 2 mo of frozen storage (Table 2). Surimi gels with 9% cryoprotectants had lower ($P<0.05$) b* values but higher whiteness than those with 0, 3, and 6% cryoprotectants. This may have been due to sugar and sorbitol, additives which decreased the level of protein content and subsequently decreased yellowness. Park (1995) also reported that higher protein contents in surimi gels resulted in less lightness and more yellowness: L* values decreased by 2.5 units, while b* values increased by 2 units as protein concentration increased by 7.5%.

Table 2—Color of Pacific whiting surimi gels prepared before and after freezing as affected by level of cryoprotectants

%Cryoprotectants	Days of freezing	L*	a*	b*	Whiteness
0	0(Fresh)[h]	82.04[abc]	−2.45[a]	5.54[a]	65.42[a]
	1	82.93[a]	−2.48[a]	5.49[a]	66.46[ab]
	14	82.97[a]	−2.42[a]	5.49[a]	66.50[ab]
	60	81.72[abc]	−2.34[a]	5.27[ab]	66.43[ab]
3	0(Fresh)	82.69[abc]	−2.39[a]	4.84[bc]	68.17[cd]
	1	81.97[abc]	−2.63[ab]	4.56[cd]	68.29[cdef]
	14	82.49[ab]	−2.44[a]	5.02[b]	67.43[bc]
	60	81.97[bc]	−2.51[a]	4.58[cd]	68.24[cde]
6	0(Fresh)	82.37[abc]	−2.61[a]	4.59[cd]	68.62[cdef]
	1	81.54[bc]	−2.73[ab]	4.43[cde]	68.25[cde]
	14	81.54[bc]	−2.56[a]	4.31[def]	68.61[cdef]
	60	81.28[c]	−2.68[ab]	4.45[cd]	67.93[d]
9	0(Fresh)	82.32[abc]	−2.80[ab]	3.99[f]	70.35[g]
	1	82.35[abc]	−3.14[b]	4.00[ef]	70.35[g]
	14	82.04[abc]	−2.76[ab]	4.00[ef]	70.04[fg]
	30	81.99[abc]	−2.67[ab]	4.00[ef]	69.99[defg]

[a-g] Means in the same column followed by different superscripts are significantly (P<0.05) different within the same color parameter.
[h] 0(Fresh) indicates gels were prepared before freezing.

Comparison between fresh and frozen surimi

No significant differences in shear strain values of fresh surimi gels (day 0) were observed among different levels of cryoprotectants (Fig. 4a). These results (repeated four times) supported our assumption that a rising trend of shear strain values of fresh surimi as cryoprotectants increased was due to a quality difference in each batch of fresh surimi. After freezing, less difference between strain values of fresh and frozen surimi were observed as level of cryoprotectants increased. The strain value of surimi gels with 0, 3, and 6% cryoprotectants decreased by 22%, 7%, and 3%, respectively, upon freezing from day 0 to day 1.

Shear stress of both (fresh and frozen) surimi gels decreased ($P<0.05$) as level of cryoprotectants increased (Fig. 4b). Shear stress of fresh surimi without cryoprotectants (17.5% protein) was 35.84 kPa. Fresh surimi with cryoprotectants at 3% (14.9% protein), 6% (13.1% protein), and 9% (11.6% protein) had shear stress of 25.35, 18.22, 13.42 kPa, respectively. This was due to a decrease in protein content of finished gels. Stress value and rigidity were strongly influenced by the concentration of proteins or other solids in surimi and processing conditions. Strain however is affected mainly by protein quality, such as type (sarcoplasma, myofibril, stroma), pH, and the degree of denaturation of proteins (Park, 1994b; Lanier, 1986; Howe et al., 1994).

Freezing reduced shear stress values of surimi gels at all 4 levels of cryoprotectants (Fig. 4b). Without cryoprotectants, the stress values of frozen surimi was reduced by 45%. A reduced stress value of 21–25% was observed in frozen surimi gels with 3 and 6% cryoprotectants, and a 19% decrease in stress value was found in frozen surimi gels with 9% cryoprotectants. The most striking difference between fresh and frozen surimi was noted in stress values. The stress value of fresh surimi with no cryoprotectants (35.84 kPa) was almost three times higher than frozen surimi with 9% cryoprotectants (11 kPa) at the same moisture level (Fig. 4b). This clearly showed the advantage of fresh surimi over commercial frozen surimi.

CONCLUSION

FROM THE RESULTS of APC, shear stress, shear strain, and color values, the use of fresh Pacific whiting surimi can be feasible for up to 5 days shelf-life at refrigerated temperature (5°C). Fresh Pacific whiting surimi also exhibited high gel-forming ability and maintained quality up to 5 days of storage.

ACKNOWLEDGEMENT

This study was financially supported by contract No. ST93.124-5628-12 from National Coastal Resources Research & Development Institute (Portland OR).

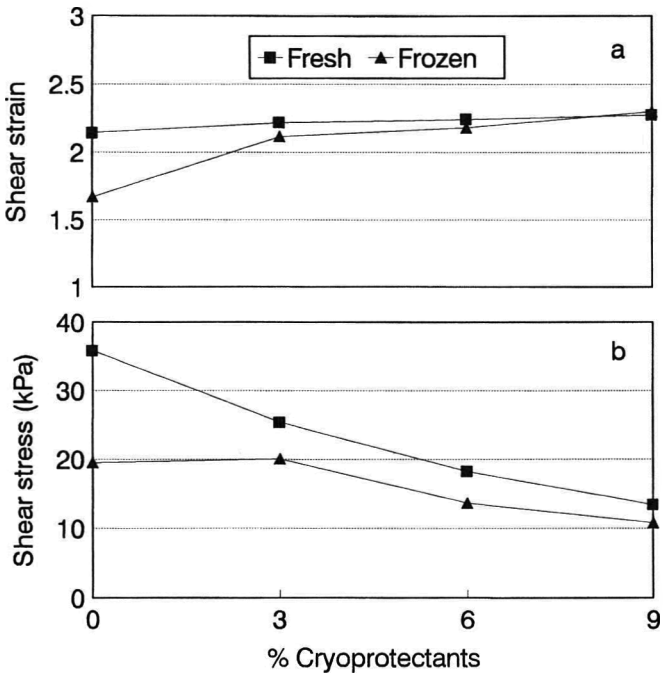

Fig. 4—Shear strain and shear stress of fresh Pacific whiting surimi gels at various levels of cryoprotectants before (day 0), and after freezing for 20–24 hr (day 1).

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Ms received 2/13/95; revised 5/1/95; accepted 5/22/95.

This research was supported in part by the USDA under Grant No. 93-34307-8371; the State of Oregon Economic Development Department Regional Strategies Program; and contract No. ST93.124-5628-12 from National Coastal Resources R&D Institute (Portland, OR).

Northern Squawfish (*Ptychocheilus Oregonensis*) for Surimi Production

D. LIN and M.T. MORRISSEY

ABSTRACT

Surimi was made from freshwater Northern squawfish (*Ptychocheilus oregonensis*) and tested for processing characteristics and quality. The surimi yield (two washes) averaged 17.9% with moisture 72.10% and protein content 17.54%. Stress and strain results indicated that it was feasible to produce surimi from squawfish stored in ice for up to 9 days. Fish freshness affected initial surimi gel strength and color and had variable effects on loss of gel strength during frozen storage.

Key Words: surimi, squawfish, gel strength

INTRODUCTION

SQUAWFISH has long been considered an underutilized species. The majority of capture is converted into fish meal and liquid fertilizer (Hanna and Pampush, 1992). The large number of small, barbed bones in the muscle are a major reason for the lack of markets for human consumption. Surimi is a minced and washed fish flesh stabilized by cryoprotectants. It is used as an intermediate raw material for manufacture of a variety of fabricated seafood products; such as, imitation crab meat, scallops, shrimp, fish sausage, and fish ham (Buck and Fafard, 1985; Lee, 1984). With an increase in consumption of surimi-based analog products several species are being considered for surimi production (Spencer et al., 1992, Kano, 1992).

A key property of surimi is its ability to form strong cohesive gels (Lanier, 1986). There is a general decline in cohesiveness and hardness of surimi gels made from fish stored over time in ice (Hamann and MacDonald, 1992). The rate at which loss of gel strength occurs appears to vary among species. The gel strength of kamaboko, a fine-textured white elastic Japanese fish cake, made from lizardfish stored on ice for 3 days was 50% of that made from fresh fish (Kurokawa, 1979). MacDonald et al. (1990) reported that gel strength of hoki stored on ice up to 10 days compared favorably with gels made from other commercial species. Morrissey et al. (1992) reported that a 24-hr delay in processing Pacific whiting could have notable impact on final surimi quality. The gel strength of Pacific whiting was reduced 10–20% for each day of iced storage. Sardines were useable for surimi for only 1 day after capture and Pacific mackerel and white hake for 2 days (Kawamura and Hasegawa, 1982; Hashimoto et al., 1987; Ablett et al., 1991). Initial studies on squawfish showed that they maintained moderately good gelling characteristics over a 2 wk period (Lin and Morrissey, 1994). Our objective was to investigate the feasibility of using squawfish, kept in ice over an extended period, for surimi processing. Processing parameters which would provide acceptable gel forming ability, color attributes and storage life for squawfish surimi were also investigated.

MATERIALS & METHODS

Fish source and storage

Fresh Northern squawfish were gathered at dam sites along the Columbia River through the Sport Bounty Program in July, 1992. Fish (≈400 kg) were packed in ice after capture and transported to the OSU Seafood Laboratory, Astoria, OR. Care was taken to ensure that all fish were delivered less than 24 hr after harvest. Fish were packed in ice chests with three layers of fish per chest. Each layer of fish was covered with a layer of ice. Excess water was continuously drained and additional fresh ice was added only to the top layer of fish during storage. The chests of iced fish were held in a thermostatically controlled cold room (4°C). At intervals of 0, 3, 6, 9, and 13 days, about 70 kg fish were taken from storage for surimi processing.

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Surimi processing

Fish were planked (separation of musculature and bone from head, backbone, tail and viscera) by hand and washed to remove remaining slime, scales, blood and adhering viscera tissue. Muscle was separated from bones and skin and minced in an Akashi deboner (Model 805, Akashi Tekkosho, Ltd., Japan). The belt tension was adjusted to remove skin from plank and to optimally recover light-colored flesh. The minced flesh was washed in polyethylene tanks with water and ice at one part flesh to three parts water (w/w) and gently stirred for 5 min, followed by holding for 5 min. The washed mince was dewatered in a Sano-Seisakusho screw press (Model SD-8, Akashi Tekkosho, Ltd., Japan). The resultant washed mince was refined with an Akashi strainer (Model S-1, Akashi Tekkosho Ltd., Japan) to separate white meat from connective tissue, bone particles and skin. Surimi was prepared by mixing the refined mince with cryoprotectants, 4.0% sucrose (C&H Pure Cane Sugar, CA), 4.0% sorbitol (ICI Specialties, New Castle, DE) and 0.3% Brifisol 512 (instantized sodium tripolyphosphate and sodium polyphosphate, B.K. Ladenburg Corp., Cresskill, NJ) in a Hobart Silent Cutter (Model VCM, Hobart Manufacturing Co., Troy, OH). Product temperatures were maintained near or below 10°C. Aliquots of 500g surimi were packed into individual plastic trays, vacuum packed, blast frozen overnight, and stored at -18°C for further analyses. The yield of surimi production was determined after each processing step.

Chemical analyses

Fish freshness was determined by K value measurement on high pressure liquid chromatography using the modified method of Ryder (1985). The proximate composition of surimi and intermediate products (mince, washed/pressed mince, refined mince and surimi) was determined in triplicate, using procedures described by the AOAC (1975).

Gel preparation

Minced flesh, washed/pressed flesh, fresh surimi and partially thawed surimi samples were used for preparation of all gels. The moisture content of samples for gel preparation was determined using a microwave procedure developed at the OSU Seafood Laboratory (Morrissey et al., 1993). All formulations for cooked gels were standardized at 2% salt and 78% moisture. Surimi paste was made by chopping 800g sample with ice and NaCl in a Stephan Vacuum Chopper/Mixer (Model 5289, Stephan Machinery Corp., Columbus, OH). All ingredients were added simultaneously and chopped for 1 min at low speed and chopped continuously for another 3 min under vacuum to form a paste with a final temperature of 5–7°C. After chopping, the paste was transferred to a sausage stuffer (Model 14208, The Sausage Maker, Buffalo, NY). Surimi paste was extruded into stainless steel cooking tubes (length 17.5 cm, i.d. 1.0 cm) coated with PAM® cooking spray (Boyle-Midway, Inc., New York, NY). The tubes were sealed with screw-on brass caps at one end and rubber caps fastened with clamps at the other end. The tubes were placed in a water bath and cooked at 90°C for 15 min. After cooking, the tubes were cooled for 15 min in iced water, and the gels were then

Table 1—Mean composition (% wet wt) of flesh derived from surimi processing unit operations[a,b]

Samples	Moisture	Protein	Lipid	Ash
Deboned	77.94±0.07	17.91±0.50	3.23±0.01	1.22±0.02
Flesh, first wash	79.23±0.02	17.73±0.24	2.58±0.05	0.65±0.01
second wash	78.74±0.05	18.86±0.08	2.51±0.04	0.61±0.05
third wash	80.48±0.08	17.38±0.33	1.96±0.04	0.50±0.01
Refined, second wash	79.48±0.07	18.54±0.05	1.78±0.03	0.48±0.01
third wash	80.32±0.09	17.98±0.22	1.61±0.04	0.42±0.01
Surimi, second wash	72.10±0.05	17.54±0.10	1.56±0.03	0.80±0.02
third wash	72.95±0.06	16.90±0.12	1.21±0.02	0.83±0.01

[a] Samples were determined in triplicate.
[b] Surimi was composed of washed and refined mince mixed with 4% sucrose, 4% sorbitol and 0.3% polyphosphates as cryoprotectants.

Table 2—Yield of flesh from squawfish surimi processing unit operations[a]

	Percent of round weight (%)					
Samples	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Mean ± S.D.
Round fish	100.0	100.0	100.0	100.0	100.0	100.0±0.0
Planked	42.8	39.6	37.7	40.8	35.1	39.2±2.6
Minced flesh	26.4	31.4	24.2	29.2	26.3	27.5±2.5
Washed flesh	17.4	20.0	15.3	18.5	15.3	17.3±1.8
Refined	16.0	19.8	14.0	17.1	14.2	16.2±2.1
Surimi	17.9	21.6	16.2	18.5	15.5	17.9±2.1

[a] Surimi samples were processed with 2 wash cycles at 1:3 mince:water ratio.

removed. The gels were stored overnight at 4°C in Whirl-Pak bags (Nasco, Fort Atkinson, WI).

Torsion test

Gel samples were removed the following day from chilled storage and allowed to reach room temperature (≈23°C) for 2 hr. Samples were cut into 2.8 cm length. Six subsamples were used for each test. The ends of the samples were blotted dry and glued with cyanoacrylate glue (Krazy Glue Inc., Itasa, IL). All samples were shaped into an hourglass form with a minimum diameter of 1.0 cm by a torsion cutter (Model 91, Gel Consultants, Raleigh, NC). Each hourglass-shaped sample was then placed in a modified torsion apparatus composed of a Brookfield digital viscometer (Model 5XHBTD, Brookfield Engineering Laboratories, Inc., Stoughton, MA). The texture of each gel was then measured by twisting the sample at 2.5 rpm until structural failure occurred (Kim et al., 1986). Torque and angular displacement were recorded on a strip chart by a Gould chart recorder (Model 154328-10, Gould, Inc., Cleveland, OH). Shear stress and true strain were calculated as described by Hamann (1983).

Color evaluation

Color of the gels was measured using a Minolta Chroma Meter (Model CR-300, Minolta Camera Co. Ltd., Osaka, Japan) with L*, a*, and b* as color coordinates. The instrument was calibrated using a standard white plate and a standard hitching tile (perfect diffuse reflector; L*=82.13; a*=−5.24; b*=-0.55) (Hunter Associate Laboratory, Inc., Reston, VA) recommended by Surimi Technical Committee of National Fisheries Institute (NFI, 1991). Whiteness was calculated as L*-3b* as described by Park (1994).

Statistical analysis

Data were analyzed for significant differences by using one-way analysis of variance. A minimum of six samples were used for all torsion and color measurements. Differences among means were tested by Least Significant Difference (LSD) multiple range test at $p<0.05$.

RESULTS & DISCUSSION

Effect of surimi processing on composition

The proximate composition of samples from the surimi unit operation was determined (Table 1). Blood, fat, and water soluble compounds were removed during washing of the minced flesh. Notable reduction occurred in lipid and ash which were reduced by 39% and 59%, respectively, after the third wash.

Table 3—Effect of freshness of squawfish on surimi quality

Fish on ice (days)	Strain	Stress (KPa)	Whiteness
0	2.13 ± 0.04[a]	22.37 ± 0.47[a]	59.69 ± 0.62[a]
3	2.19 ± 0.02[a]	21.46 ± 0.70[a]	59.70 ± 0.49[a]
6	1.97 ± 0.02[b]	14.20 ± 0.55[c]	57.48 ± 0.30[b]
9	1.90 ± 0.02[c]	17.39 ± 0.42[b]	56.83 ± 0.35[b]
13	1.86 ± 0.02[c]	15.71 ± 0.54[c]	55.26 ± 0.16[c]

[a-e] Means within a column not sharing a common letter were significantly different ($p < 0.05$). Surimi processed with two wash cycles at a 1:3 mince:water ratio.

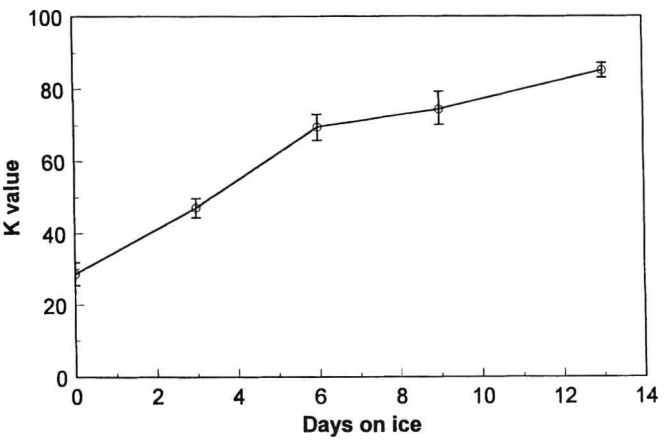

Fig. 1—Effect of iced storage time on K-values of northern squawfish.

The refining process reduced the amount of connective tissue, small skin and bone particles, further decreasing the lipid content to half the original. Washing also decreased protein content and three washes decreased the protein content below 17% for the final surimi. Preliminary studies showed no significant differences in texture and color in surimi made after the second or third wash. All subsequent surimi tests were made from mince that was washed twice and then refined. Based on two wash cycles, the proximate composition of squawfish surimi was 72.10% moisture, 17.54% protein, 1.56% fat, and 0.80% ash.

Product yield in surimi unit operations

Squawfish yielded 39.2% planks which produced 27.5% machine-separated minced flesh based upon round weight (Table 2). Two 3:1 (water:flesh) wash procedures yielded 17.3% pressed flesh which produced 16.2% refined product and 17.9% surimi (91.7% refined flesh and 8.3% cryoprotectants) based upon round fish weight.

Effects of freshness on surimi quality

Gel strength, as measured by stress and strain, was affected by length of time the fish were kept in ice before processing. Surimi made from fish stored in ice at day 0 and day 3 had the highest strain and stress values (Table 3). There were subsequent declines in both strain and stress of surimi made from fish kept on ice after the third day. By day 13, the strain and stress values of surimi gels decreased to 1.86 and 15.71 KPa, respectively.

The decline in gel forming ability of squawfish was less rapid than that described for lizard fish which showed a 50% decline in gel strength after 3 days storage in ice (Kurokawa, 1979). Morrissey et al. (1993) noted a 10–20% daily decrease in gel strength for Pacific whiting kept in ice. Studies by MacDonald et al. (1990) showed that good quality surimi could be made from hoki stored on ice for up to 10 days.

The rate of decline in gel strength is dependent on denaturation and extent of proteolysis of myofibrillar proteins. Squawfish showed negligible protease activity when assayed by

Table 4—Effect of freshness of squawfish on gel properties of surimi during frozen storage[f]

Frozen storage time (months)	True strain Fish on ice (days) before processing 0	3	6	9	13
1	2.23 ± 0.03[a]	2.22 ± 0.04[a]	2.19 ± 0.02[a]	2.23 ± 0.02[a]	1.96 ± 0.03[a]
3	2.15 ± 0.03[a]	2.09 ± 0.03[b]	2.05 ± 0.03[b]	1.98 ± 0.02[b]	1.72 ± 0.02[b]
5	2.20 ± 0.02[a]	2.14 ± 0.04[b]	2.09 ± 0.02[b]	1.96 ± 0.05[b]	1.68 ± 0.04[b]
7	2.19 ± 0.02[a]	2.12 ± 0.01[b]	2.18 ± 0.03[a]	2.00 ± 0.05[b]	1.74 ± 0.05[b]
	Shear stress (KPa) Fish on ice (days) before processing 0	3	6	9	13
1	18.38 ± 0.55[a]	16.01 ± 0.78[a]	15.88 ± 0.59[b]	15.03 ± 0.37[ab]	15.89 ± 0.54[a]
3	18.48 ± 0.29[a]	15.04 ± 0.46[ab]	15.66 ± 0.85[b]	12.74 ± 0.65[c]	11.90 ± 0.45[c]
5	16.11 ± 0.27[b]	13.58 ± 0.48[b]	14.83 ± 0.53[b]	13.72 ± 0.36[bc]	12.43 ± 0.42[bc]
7	17.27 ± 0.44[ab]	15.97 ± 0.56[a]	17.87 ± 0.66[a]	16.06 ± 0.64[a]	13.33 ± 0.40[b]

[a-e] Means within a column not sharing a common letter were significantly different ($p < 0.05$).
[f] Values represent mean values ± s.d. of six determinations of gel sample from squawfish surimi processed with two wash-cycles at 1:3 mince-water ratio.

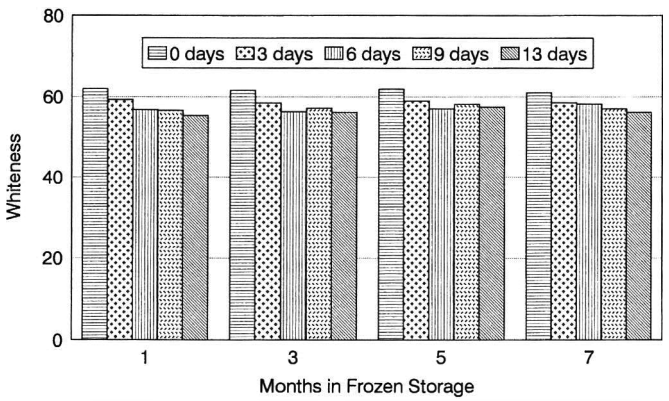

Fig. 2—Whiteness values (L* - 3b*) of squawfish surimi made from iced fish on different days and stored at −18°C.

autolysis (Morrissey et al., 1995). This may explain why the rate of gel strength loss with relation to freshness was relatively minor compared to other species. Using the minimum strain value of 1.9 for acceptable quality surimi (Hamann and Lanier, 1987), squawfish surimi could be made from fish stored in ice up to 9 days.

Significant differences were found in whiteness due to pre-processing freshness of the fish. The whiteness of the surimi samples decreased from 59–60 over the first 3 days to 55 for surimi made at day 13. L* values ranged from 79.70 to 82 and there was no discernible pattern in relation to fish freshness. The change in whiteness was due primarily to an increase in b* (yellowness) in surimi as the fish was kept longer in ice. The b* value was at 6.67 at day 0 and increased to 8.68 by day 13. Medium grade pollock surimi has a whiteness value between 65-70 (Park, 1994). The squawfish surimi was more red and yellow and slightly darker in color when compared to standard surimi color. The color of squawfish surimi could be improved using fillets instead of planks for deboning.

K value is a measure of fish freshness based on nucleotide breakdown products (Ryder 1985). The changes in K value of squawfish during iced storage are shown in Fig. 1. The K value increased rapidly in the first 6 days. After fish were stored on ice 9 days, the K value reached 74.3%. Ehira (1976) recommended that fish should not be processed once the K value exceeded 75%. Since different fish species have varying K value patterns, our results suggest that squawfish could be used for processing even though the K value reached 75%.

Effects of fish freshness on surimi quality during frozen storage

The degree of decreased strain and stress values of surimi gels during frozen storage was affected by freshness of fish at the time of processing (Table 4). There was no decrease in strain value for surimi made at day 0 and stored at -18°C for 7 mo. After 7 mo storage, the strain value of gels made from 9-day old and the 13-day old fish had decreased ≈10% from the original values. Although stress results were more variable, surimi made from 13-day old fish showed a decrease in stress values during frozen storage. Surimi made from squawfish within 24 hr had the best quality and long-term frozen storage stability. Results demonstrated that fish freshness was an important factor for production of good quality surimi. The changes in whiteness values during frozen storage were summarized (Fig. 2). As discussed, there was an inverse correlation with whiteness and fish freshness in surimi production. There were no significant differences in whiteness over 7 mo frozen storage for surimi made for each time period. Surimi made at day 0 maintained the highest values throughout frozen storage.

CONCLUSIONS

THE QUALITY OF SURIMI in terms of gel cohesiveness and gel strength, as well as whiteness, were within acceptable levels compared to other species. The washing procedure decreased fat and ash content of surimi and increased whiteness of surimi gels significantly. Fish freshness had a positive effect on surimi quality and changes during frozen storage. K value might provide the basis of a raw material quality control system for squawfish surimi production.

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Herring Surimi During Low Temperature Setting, Physicochemical and Textural Properties

JULIAN K. CHAN, TOM A. GILL, JOHN W. THOMPSON, and DOUGLAS S. SINGER

ABSTRACT

Herring surimi was held at 10°C for 0 to 24 hr, then heated at 90°C for 30 min. Rigidity, shear stress and shear strain at failure increased with holding time at 10°C, and the values of shear strain were ≥2.0 when setting time was > 3 hr. Electrophoresis revealed three types of protein-protein interactions involved in the formation of myosin polymers during setting at 10°C and/or subsequent heating at 90°C. The interactions included covalent, nondisulfide interactions; disulfide bonds and noncovalent interactions. The contributions of each type of protein-protein interaction to the formation of myosin polymers varied with setting time. Variations in the types of interactions were related to physical/chemical properties.

Key Words: herring, surimi, physicochemical, texture, low-temperature setting.

INTRODUCTION

LOW TEMPERATURE SETTING (*suwari*) is very important in production of *neriseihin* (surimi-based products) and affects the final textural strength and elasticity of fish muscle gels. Several studies have demonstrated that gel strength, breaking strength and strain of surimi gels increased with setting time between 0 and 24 hr at 10°C with or without subsequent high temperature heating (Niwa et al., 1991; Numakura et al., 1985, 1990). The gel strength of low temperature-set surimi is related to the Ca-ATPase activities associated with the myosin molecules (Funatsu and Arai, 1992; Numakura et al., 1989). Surimis with higher Ca-ATPase activities normally exhibited higher gel strengths. A positive correlation was found between the covalent cross-linking of the myosin heavy chain (MHC) and the gel rigidity in surimi from Alaska pollack and threadfin bream (Lee et al., 1990; Nishimoto et al., 1987). The formation of covalently cross-linked MHC's has sometimes been mediated by transglutaminase in some fish species (Kimura et al., 1991; Seki et al., 1990). Disulfide linkages and hydrophobic interactions have also been reported in the formation of fish myosin aggregates and/or gels (Chan et al., 1993; Itoh et al., 1979; Wicker et al., 1989).

Herring (*Clupea harengus*) is an abundant but underutilized fish species in North America and has potential for production of surimi-based products. The textural properties of herring surimi gels are undesirable when compared with other white-fleshed species which form firm and elastic muscle gels (Spencer et al., 1987). Chan et al. (1992b, 1993) compared the denaturation and aggregation behavior of cod (*Gadus morhua*) and herring myosins, and reported that the less acceptable gel forming ability of herring muscle proteins was related to the unfolding profile of interior hydrophobic domains when heated. Chan and Gill (1994) demonstrated that it was possible to improve the aggregation ability of herring myosin in a model system by blending thermally denatured herring myosins with those from cod. However, it is difficult to blend a heat-treated surimi with an unheated surimi from another fish species because preheated surimi forms a muscle gel prior to blending. Therefore, in order to denature the surimi proteins and to avoid formation of heat-set gels prior to blending, the most practical means may be through low temperature setting. Our primary objective was to investigate the physicochemical changes of myofibrillar proteins in surimi during low temperature setting at 10°C. The relationship between textural properties of surimi gels and associated chemical interactions was compared with results in a model system.

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MATERIALS & METHODS

Preparation of surimi

Herring were purchased on the same day of catch from a local fish plant. Surimi was prepared immediately upon delivery to the pilot plant. Herring were deboned in a Bibun Model SDX 16 meat/bone separator equipped with a drum with 5 mm perforations (Bibun Equipment, Hiroshima, Japan). Minced fish was processed by the traditional Japanese tank and paddle washing procedure (Suzuki, 1981) but three washes with a water:minced fish ratio of 3:1 were used. Wash water containing 0.3% NaCl (w/w) was precooled to 3–4°C. Sodium erythorbate (0.2%, w/w) was added to the wash water as an antioxidant. The washed mince was then dewatered in a basket centrifuge and refined with a 2 mm Bibun Model 420 strainer. Cryoprotectants [4% sucrose (w/w); 4% sorbitol (w/w) and 0.3% sodium tripolyphosphate (w/w)] were mixed with the dewatered mince, and then vacuum packed, quick frozen in a plate freezer, and stored at −20°C for varying periods of time.

Proximate analysis of herring surimi

Protein (Kjeldahl nitrogen), lipid and moisture contents of herring surimi were determined as described by Woyewoda et al. (1986). They were 13.77 ± 0.07% (n=3), 1.75 ± 0.06% (n=3) and 70.9 ± 0.4% (n=3), respectively.

Preparation of fish gels

Fish gels were prepared as described by Lanier et al. (1991). Briefly, surimi was removed from frozen storage and tempered overnight at 4°C, then cut into small cubes and homogenized with 2.5% NaCl (w/w) in a vacuum cutter/mixer (Stephan VCM12, Stephan u. Sohne GmbH & Co., Hameln, Germany), until a temperature of 5°C was reached. The fish paste was then stuffed into cylindrical steel tubes (20 × 200 mm) with a manual sausage stuffer (F.Dick, GmbH, Esslingen, Germany). Ends of sample tubes were sealed with crown-type bottle caps. Samples were held in an incubator for varying time periods at 10°C for low temperature setting, heated in a water bath at 90°C for 30 min, cooled immediately in ice water and stored at 4°C.

Torsion tests

Samples were allowed to equilibrate to 21°C prior to milling into dumbbell-shaped specimens as described by Lanier et al. (1991). Specimens had a minimum center diameter of 10 mm, length of 28.7 mm and an end diameter of 18.6 mm. Ends of samples were glued to plastic disks (Piedmont Plastics, Charlotte, NC) with cyanoacrylate adhesive, and mounted on a torsion apparatus consisting of a Brookfield Model DV-I digital viscometer (Brookfield Engineering Laboratories Inc., Stoughton, MA). The viscometer was operated at 2.5 rpm and the output was recorded on a strip chart recorder. Shear stress and strain were calculated using equations proposed by Hamann (1983) and rigidity was derived by dividing shear stress by shear strain at failure.

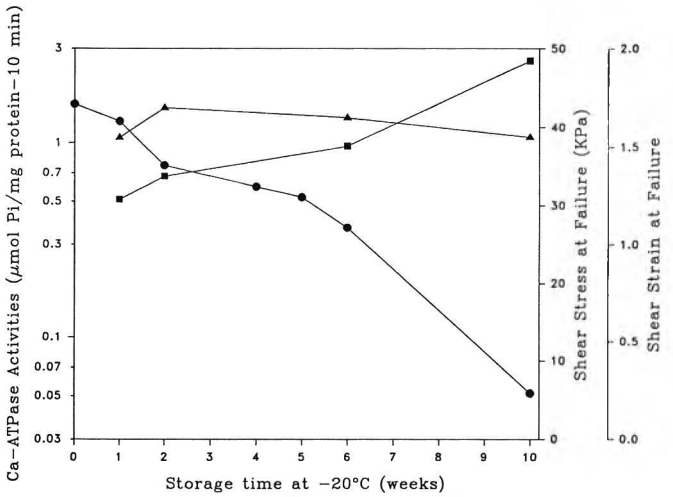

Fig. 1—Effect of storage at −20°C on Ca-ATPase activities (●) of uncooked surimi and the shear stress at failure, kPa (■) and shear strain at failure (▲) of herring surimi cooked at 90°C for 30 min. Each point is the mean of at least three determinations, and error bars representing ± 1 standard derivation are too small to be seen.

Dynamic oscillatory tests

Fish paste was prepared as described and stuffed into cylindrical tubes 40 x 60 mm (i.d. × length). The samples were kept in a 10°C incubator for 0, 3, 6, 9, 12, and 24 hr. After setting, the cylindrical samples were pushed out of the tubes into a steel O-ring (40 × 1 mm, i.d. × height), and sliced to provide wafer-shaped specimens with dimensions 40 × 1 mm (diam × height). Specimens were placed on the thermocontrolled heating platform of a Bohlin VOR Rheometer (Bohlin Rheology Inc., Edison, NJ) and rheological properties were measured by using parallel plates (40 mm in diameter) and at a constant frequency of 1 Hz and maximum oscillatory strain of 0.02. Each specimen was heated from 15 to 80°C at 2C°/min and dynamic shear measurements were recorded at regular intervals. Samples between parallel plates were covered with a plastic chamber (supplied by manufacturer) to prevent drying.

Ca-ATPase activities

Fish paste (10g) which had been set at 10°C for a predetermined time, was homogenized with an ice-chilled buffer containing 0.1M KCl, 5 mM $CaCl_2$ and 25 mM Tris-maleate (pH 7.0) in a Lourdes MM1B mixer (Verntron Medical Products, Inc., Carlstadt, NJ) for 2 min, full power. The homogenate was diluted to 100 mL with buffer, then 1 mL of diluted protein suspension was mixed with 49 mL buffer. The concentration of final protein suspension was 0.2 to 0.3 mg protein/mL as determined by the Biuret test using bovine serum albumin as a standard. The reaction medium contained 2.4 mL final protein suspension, 2.4 mL buffer and 0.2 mL 25 mM ATP solution (freshly prepared, dissolved in the buffer); and was incubated in a water bath at 25°C for exactly 10 min. The reaction was stopped by addition of 1.25 mL 20% trichloroacetic acid. The sample was centrifuged in a bench top centrifuge for 10 min, and the inorganic phosphate (Pi) content of the supernatant was determined as described by Fiske and Subbarow (1925). Ca-ATPase activity of each sample was calculated by subtracting the Pi content of the sample from the controls and expressed as μmol P_i/mg protein-10 min.

ANS binding studies

Samples of fish paste (10g), which had been set at 10°C for a predetermined time, were homogenized with an ice-chilled buffer containing 0.3M KCl, 10 mM sodium phosphate (pH 7.0) in a Lourdes MM1B mixer (Vemtron medical products, Inc., Carlstadt, NJ) for 2 min, full power. The homogenate was diluted to 100 mL with buffer, then 1 mL of diluted protein suspension was mixed with 49 mL buffer. The concentration of the final protein suspension was about 0.2 to 0.3 mg protein/mL. The exact concentration was determined by Biuret test using bovine serum albumin as a standard. Fluorescence intensity was measured immediately upon addition of 15 μL of 8 mM 8-anilino-1-naphthalene sulfonic acid (ANS) (BDH Ltd., Poole, England) to 3 mL of the protein suspension (0.05 to 0.2 mg/mL). Fluorescence intensity was recorded with a Perkin-Elmer LS50 luminescence spectrometer; the PMT voltage was kept at 750V throughout the experiment. Excitation and emission wavelengths were 390 nm and 470 nm, respectively; the excitation and emission slits were 5 nm. Surface hydrophobicity was determined as the initial slope of a plot of fluorescence intensity vs. protein concentration (Li-Chan et al., 1985).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples of surimi gels in torsion tests were subjected to electrophoresis to monitor chemical interactions in the gelation process. Surimi gels (0.2g) were mixed with 5 mL 25 mM Tris/HCl (pH 7.4) containing 2.5% SDS (w/v), 8M urea, with or without 2% β-mercaptoethanol (v/v), and boiled at 90°C for 5 min followed by continuous stirring for 24 hr at ambient temperature. The samples were then centrifuged at 2,000 × *g* for 10 min to remove insoluble materials (mostly pigments) and dialyzed against three changes of 0.1% SDS (w/v), 25 mM Tris/HCl (pH 7.4) with or without 0.2% β-mercaptoethanol (v/v) in a volume ratio of 1:100 (sample:dialysis buffer) at room temperature within 24 hr. The dialysis tubing was Spectra/Por® regenerated cellulose membrane, a molecular weight cut-off 12,000–14,000 (Spectrum, Houston, TX). The protein concentrations of the dialyzed samples were determined by the Bradford (1976) assay using bovine serum albumin as a standard. Samples (30 μg/lane) were subjected to electrophoresis on cylindrical 5 mm × 115 mm 7.5% polyacrylamide gels as described by Porzio and Pearson (1977). After staining with Coomassie Brilliant Blue R250, gels were destained and scanned densitometrically at 555 nm on a PU 8800 spectrophotometer (Pye Unicam, Cambridge, England) with gel scanning and peak integration equipment. The integrated peak area for myosin was expressed as a percentage of total peak area for all myofibrillar proteins. Student's t tests were used to compare results from two treatments and one way analysis of variance was used to compare means of more than two treatments (Li, 1966).

Differential scanning calorimetry (DSC)

Fish paste (50 60 mg) was sealed in Perkin-Elmer stainless steel sample pans (Perkin-Elmer Corp., Norwalk, CT). An empty pan was used as reference. Samples were heated at 5 °K/min from 283 to 363°K in a Perkin-Elmer DSC 2C differential scanning calorimeter.

RESULTS & DISCUSSION

Effect of frozen storage

The average Ca-ATPase activities of native herring surimi (freshly prepared, non-frozen) were 1.57 μmol P_i/mg protein −10 min (Fig. 1) The Ca-ATPase activities of frozen surimi decreased gradually with storage time at −20°C and were reduced nearly by 2 log cycles after 10 wks. DSC results showed that the T_{max} and T_{onset} values for myosin in the native surimi were 47.25 ± 0.94°C (n=3) and 43.72 ± 0.79°C (n=3) (T_{max} thermal transition and T_{onset} temperature at beginning of transition). After 1 wk at −20°C, the T_{max} and T_{onset} of myosin were lower ($p < 0.05$) than for native surimi: 43.16 ± 0.15°C (n=3) and 36.77 ± 0.07°C (n=3), respectively, suggesting some denaturation of myosin as a result of frozen storage. SDS-PAGE of native surimi revealed that myosin heavy chains (MHC) decreased slightly in intensity and formed polymers (mainly dimers) during the first several weeks of frozen storage. The amount and/or type of cryoprotectant we used did not completely prevent denaturation and aggregation of myosin molecules in herring surimi during frozen storage.

In the case of surimi gels heated at 90°C for 30 min without low temperature setting at 10°C, the torsional stress at failure increased by ~40% with storage time at −20°C, but there was no discernible difference in torsional strain at failure during frozen storage (Fig. 1). We could not determine whether the increase in torsional stress was caused by frozen storage denaturation and/or cross-linking of myosin in the herring surimi. In order to study the effect of low temperature setting on physical and chemical properties of surimi, subsequent experiments were performed on samples taken on the second and sixth weeks of frozen storage.

Torsion tests

Shear stress and shear strain at failure are reported to be measures of strength and cohesiveness of muscle gels, respectively. Rigidity refers to firmness and brittleness of surimi gels. Gel cohesiveness (strain) is the most sensitive and important parameter to describe protein quality and functionality of surimi (Lanier, 1986). Hamann et al. (1990) proposed that good quality surimi with respect to gel cohesiveness should have a true strain value >2.0. Our results, the true strain at failure of surimi gels increased with setting time at 10°C, and its value was >2 when the setting time was ≥6 hr. The shear stress at failure of the herring surimi gels also increased with setting time at 10°C and seemed to increase more rapidly in the first 3 hr of setting (Fig. 2).

A textural map (Fig. 3) of herring surimi gels was proposed by Lanier et al. (1991). "Mushy" implies that a gel sample has little integrity, and "tough" refers to high resistance to force combined with large deformation prior to fracture. "Rubbery" refers to high deformation with low rigidity and brittle is low deformation and high rigidity. Note that the position of these terms is adopted from Lanier et al. (1991) and does not represent the actual sensory-textural relationship of tested herring surimi gels. Nevertheless, rigidity of herring surimi gels increased rapidly with setting time at 10°C, while the cohesiveness (strain) also gradually increased with setting time. Note that the increment for rigidity was much greater than for shear strain at failure of surimi gels when setting time was >6 hr. Thus, our results showed that the textural properties of herring surimi gel could be improved by setting at 10°C first, followed by high temperature heating.

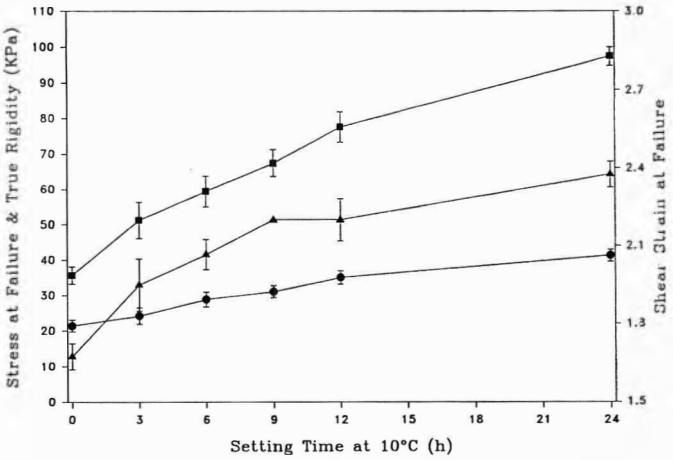

Fig. 2—Effect of setting time at 10°C on stress and strain at failure of herring surimi gels. Gels were subsequently heated at 90°C for 30 min after the predetermined setting time. (■) Shear stress at failure; (▲) shear strain at failure; and (●) rigidity. Each point is the mean of six determinations, and error bars represent ± 1 standard derivation.

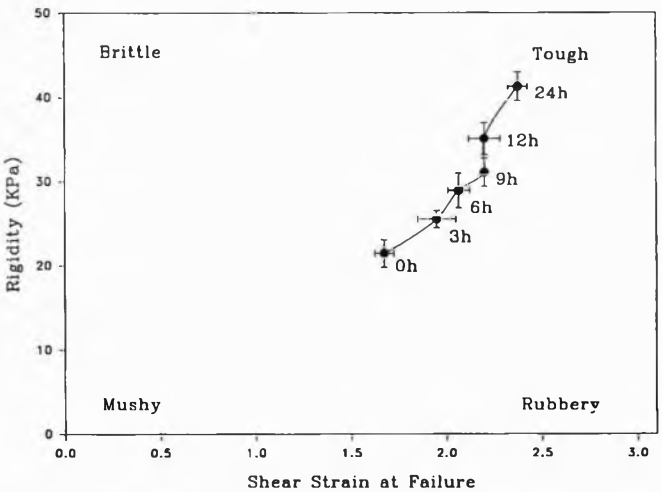

Fig. 3—Textural map of herring surimi gels. The numerical figures represent setting times at 10°C. Each point is the mean of six determinations, and error bars represent ± 1 standard derivation.

Dynamic oscillatory tests

Storage modulus, G', is a measure of energy recovered per cycle of sinusoidal shear deformation and is defined as stress in phase with strain divided by strain. Loss modulus, G", is an estimate of energy dissipated as heat/cycle and is defined as stress out of phase with strain divided by strain in sinusoidal deformation. The ratio of loss and storage moduli is numerically equal to the tangent of the viscoelastic phase angle and is a measure of energy lost compared to energy stored in cyclic deformation (Ferguson and Kemblowski, 1991). When the phase angle approaches 0 degrees, negligible energy is lost as heat and the material is predominantly elastic in nature. A phase angle of 90° indicates a negligible energy recovery and the fluid is predominantly viscous. Our objective in employing the dynamic oscillatory test was to examine the effects of low temperature setting at 10°C on formation of protein networks developed in the herring surimi gels. The advantage of oscillatory testing is that the amplitude of oscillation is small so as not to disrupt the gel during testing. For these tests, wafers of unheated surimi held at 10°C were placed between parallel plates and rheological properties measured as temperature was increased from 15 to 80°C at 2C°/min. For the herring surimi without setting, the G' and G" started to decrease to a minimum when temperature increased from 15 to 46° (Fig.4a, b). Above 46°C, G' increased rapidly up to 66°C and then decreased slightly while G" increased only very slightly. In addition, the phase angle (δ), between input stress and measured strain oscillatory response, dropped to 6° and remained unchanged at 46°C or above, respectively. This suggested that the sample became more elastic as temperature increased <46°C. Since T_{max}, the denaturation temperature of herring myosin, as determined by DSC, was 43.72°C, the development of an elastic network in surimi gel may be related to overall conformational changes in denatured myosin molecules. The herring surimi having been held at 10°C for 3 to 9 hr, behaved in a manner similar to the nonset sample (0 hr at 10°C). The degree of elastic response generally increased inversely with duration of low temperature setting for final heating temperatures of 46°C and above. The data suggested that conformational changes of herring myosin molecules and/or new types of protein-protein interactions might take place during setting at 10°C. Its effect upon development of gel network during high temperature heating was related to and increases with setting time.

Ca-ATPase activities and ANS binding

Ca-ATPase activity is a measure of muscle tissue ability to hydrolyze adenosine triphosphate in the presence of Ca^{2+} ions. The myofibrillar adenosine triphosphatase (ATPase) is located in the myosin head region. Takahashi et al. (1962) found that the helical content of myosin molecules decreased by only a few percent even after its ATPase activity completely disappeared. Hence, the loss of ATPase activity does not necessarily imply that the myosin head has been unfolded. It only indicates that myosin heads undergo some conformational changes. The fish pastes lost their Ca-ATPase activities rapidly in the first several hours of setting and were reduced to about 50% and 12% of the initial value after 12 and 24 hr incubation, respectively (Fig. 5).

It has been generally hypothesized that proteins fold so as to pack the apolar domains in a nearly crystalline state. Denatur-

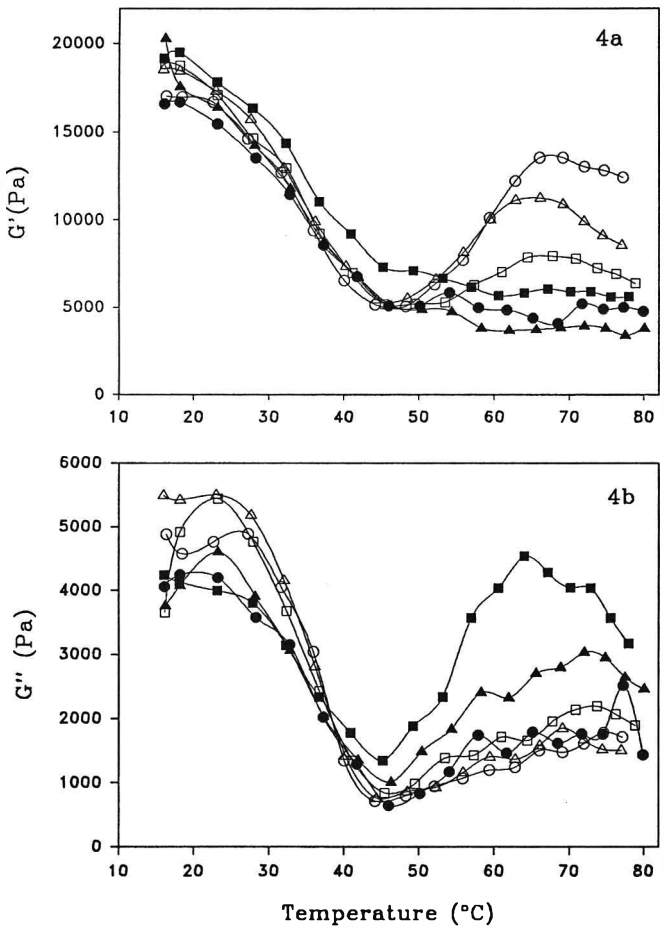

Fig. 4—Effect of setting time at 10°C and temperature rise on storage modulus (G', 4a) and loss modulus (G", 4b) of surimi gels during heating from 15 to 80°C at a rate of 2C°/min. (○), 0 hr; (△), 3 hr; (□), 6 hr; (●), 9 hr; (▲), 12 hr; and (■), 24 hr setting time at 10°C. Each point is the mean of two determinations.

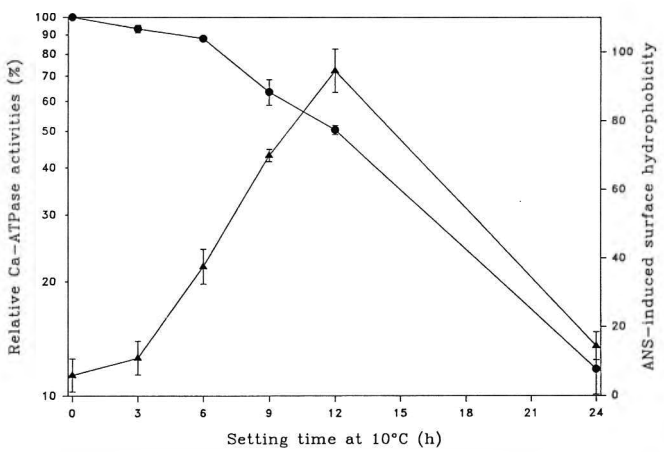

Fig. 5—Changes in Ca-ATPase activities and ANS induced surface hydrophobicity of herring surimi during setting at 10°C. (●), Ca-ATPase activities; and (▲) surface hydrophobicity. The Ca-ATPase activity was calculated as the proportion of detected activities at time t to initial activities at time 0 (expressed as percentage) in each experiment. Each point is the mean of four determinations, and error bars represent ± 1 standard derivation.

ation often results in proteins unfolding their interior hydrophobic surfaces. The ANS induced surface hydrophobicity of myofibrillar proteins increased slightly during the first 3 hr at 10°C (Fig. 5) but increased to a maximum for setting times of 12 hr. After 24 hr incubation at 10°C, the surface hydrophobicity dropped to a value similar to that at time 0. Surimi is mainly composed of myofibrillar proteins with myosin 43% and actin 22% of the total. In general, the thermal stability of actin is higher than for myosin. Chan et al. (1992b) reported that fish myosin lost its helical content with simultaneous exposure of interior hydrophobic surface when the proteins were thermally denatured. Thus, it is reasonable to hypothesize that the discernible increase of surface hydrophobicity in surimi during 10°C setting is due to the unravelling of myosin molecules. The results of Ca-ATPase activity and ANS binding studies suggested that herring myosin molecules underwent some conformational changes during setting at 10°C, and the rates of these changes appeared to be slow initially and then to increase when setting time was extended.

We could not explain why the ANS induced hydrophobicity decreased in the fish pastes being set for more than 12 hr (10°C). One explanation may be that after 12 hr at 10°C, some protein-protein interactions occluded the formerly exposed hydrophobic domains, making them inaccessible to the ANS hydrophobic probe.

SDS-PAGE

SDS-PAGE is commonly used to study chemical interactions in gelation of muscle proteins. Urea and SDS, an anionic detergent, can dissociate any noncovalent interactions present in the muscle proteins. The presence of protein polymers in urea and SDS suggests the involvement of only covalent bonds. Surimi gels (90°C, 30 min) prepared at different 10°C setting times were examined by SDS-PAGE in the presence or absence of β-mercaptoethanol to determine the involvement of disulfide bonds. The 10°C setting time had no effect on band intensities for actin or any other components with molecular weights <45,000 Da. The densitometric intensities of MHCs accounted for 43% of total protein in the samples of purified herring myofibrils and in freshly prepared surimi. However, once the surimi was frozen, the amount of MHC was reduced by 5%, and the MHC was polymerized mostly as dimers. In the case of surimi gels (90°C, 30 min) without any setting at 10°C, the relative amount of MHC was about 37.99 ± 2.93% (n=4) in the presence of β-mercaptoethanol and 27.14 ± 1.81% (n=4) in its absence. When surimi gels were solubilized in sample buffer containing β-mercaptoethanol, the MHC content in the surimi gels decreased rapidly (Fig. 6) with setting times for the first 9 hr; then decreased slightly for setting times >9 hr. Without β-mercaptoethanol, the MHC content decreased rapidly for setting times ≤3 hr, and then remained unchanged for setting times up to 24 hr, at which time the MHC only accounted for 4% of total proteins.

Electrophoretic results provided evidence for at least four types of protein-protein interactions involved in formation of MHC polymers during setting (10°C, 0–24 hr) and/or heating (90°C, 30 min). The data support the existence of covalent non-disulfide interactions, disulfide bonds, noncovalent interaction(s), as well as evidence for the formation of cross-linked MHCs during frozen storage of cryoprotected frozen surimi.

The relative extent of each of the interactions was estimated by measuring the densitometric intensity of protein bands on an electrophoretic gel. The relative amount of monomeric MHC (I) in a sample was calculated from the ratio of intensity of MHC band to intensities of all protein bands present on the electrophoretic gel. SDS-PAGE in the presence of β-mercaptoethanol showed that MHCs accounted for 43% of the total proteins in the purified herring myofibrils and in freshly prepared surimi. Thus, any reduction of densitometric intensity for MHCs (<43% of total proteins) indicates that MHCs are either cross-linked by some protein-protein interaction or that proteolytic degradation of MHC molecules is considerable. The amount of MHC decreased after the surimi was frozen. The relative amount of MHC (%) being cross-linked through covalent nondisulfide bonds during frozen storage was estimated by $\{(43\% - I_{t=0,+me})/$

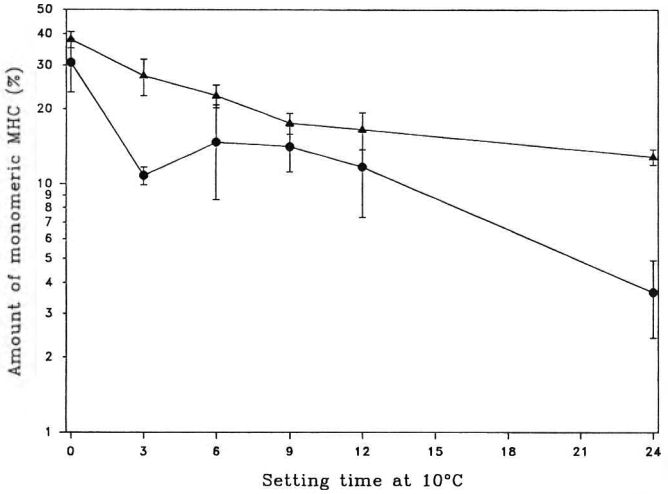

Fig. 6—Changes in the amount of monomeric myosin heavy chains in herring surimi gels which were heated at 90°C for 30 min after the predetermined setting time at 10°C. (●) Gels solubilized in sample buffer containing 2.5% SDS (w/v), 8M urea, 25 mM Tris/HCl (pH 7.4); (▲) gels solubilized in the same sample buffer but with 2.5% β-mercaptoethanol. Each point is the mean of four determinations, and error bars represent ± 1 standard derivation.

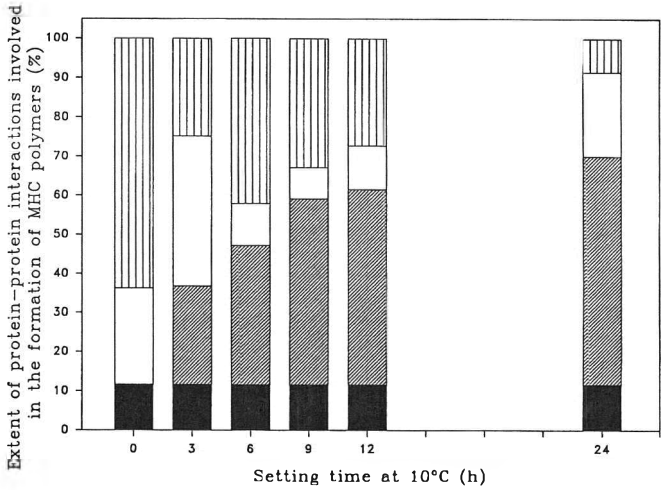

Fig. 7—Extent of different types of protein-protein interactions in formation of myosin heavy chain (MHC) polymers in surimi gels. The results based on densitometric scanning of SDS-PAGE gels and calculations for the extent of each protein-protein interaction are discussed in the text. (■) Cross-linking of MHC induced by frozen storage; (▨) covalent, non-disulfide interactions; (□) disulfide linkages; and (▥) noncovalent, hydrophobic interaction.

43%} × 100%, where I is the relative amount of MHC (%) determined by SDS-PAGE; t is the setting time (hr) at 10°C; and "+ or − me" refers to the presence or absence of β-mercaptoethanol. The relative amount of MHC (%) being cross-linked by covalent nondisulfide interactions during setting at 10°C was estimated by $\{(I_{t=0,+me} - I_{t,+me})/43\%\} \times 100\%$; assuming that reduction of the MHCs densitometric intensity in the surimi samples with different setting times indicated the proportion of MHC polymers being insolubilized in the buffer containing SDS, urea and β-mercaptoethanol. Estimates for the amount of MHC (%) being cross-linked by disulfide bonds were based on differences in MHC's densitometric intensities of a surimi sample in the presence and absence of β-mercaptoethanol and expressed as $\{(I_{t,+me} - I_{t,-me})/43\%\} \times 100\%$. We assumed that the densitometric intensity of the MHC in surimi samples solubilized in the SDS buffer without β-mercaptoethanol reflected the amount which was not cross-linked by any covalent protein-protein interactions.

Without low temperature setting, most of the covalent interactions in surimi gels involved disulfide bonds (Fig. 7). The relative extent of disulfide bonding increased sharply in the first 3 hr setting at 10°C, then became reduced at 6, 9, and 12 hr as compared to 3 hr. After 24 hr incubation, the extent of disulfide linking increased from about 11 to 21%. The accessible cross-linking sites on MHC molecules appeared to be limited and a reduction in extent of disulfide bonding may be due to a concomitant increase of covalent nondisulfide interactions (Fig. 7). There are about 42 SH groups in a myosin molecule and two highly reactive thiols are present in each myosin head. Chemical modification of these two thiols alters the ATPase activities of myosin (Harrington and Rodger, 1984). Itoh et al. (1979) reported that the rigidity modulus of carp actomyosin gels formed at 40 and 80°C was decreased by chemically blocking the thiol groups of the fish actomyosin. In addition, a large decrease in gel strength of surimi gels from various species accompanied a decrease in the Ca-ATPase activities of the surimi gels during low temperature setting between 10 and 30°C (Numakura et al., 1987, 1989). The decrease of Ca-ATPase activities was coincidental with the increase in disulfide linkages. One explanation for this may be that reactive thiols in the myosin heads were exposed during 10°C setting and underwent oxidation to form intermolecular S–S bonds, most probably during subsequent heating at 90°C.

The covalent nondisulfide interactions involved in formation of MHC polymers increased rapidly with setting time at 10°C (Fig. 7). Seki et al. (1990) hypothesized that the formation of covalently cross-linked MHC polymers during low temperature setting might be mediated by transglutaminase (TGase) present in the surimi prepared from some fish species. This hypothesis was confirmed by Kimura et al. (1991) who demonstrated the formation of ε-(γ-glutamyl) lysine isopeptide in suwari-kamaboko gels. We could reasonably assume that the observed covalent, nondisulfide linkages among the herring MHC polymers in our results were mediated by TGase during the setting period. Thus, longer incubation times at 10°C may result in a progressive unfolding of the MHC and provide more accessible lysine and glutamine residues necessary for TGase-induced cross linking. On the other hand, according to Nowsad et al. (1994a, b), the setting of surimi paste and aggregation of MHCs does not necessarily involve TGase . The final textural properties of surimi gels are most likely to involve a combination of covalent and noncovalent interactions rather than being mediated by TGase alone as was suggested in earlier studies.

Despite the potential involvement of TGase and formation of disulfide bonds in the low temperature setting of surimi, there is little doubt that noncovalent interactions were also involved in formation of MHC aggregates. Our earlier studies involving model systems showed good correlation between extent of aggregation and surface hydrophobicity of purified herring myosin solutions during heat treatments between 35 and 55°C (Chan et al., 1993). No evidence for covalent (disulfide or nondisulfide) cross linking was observed in our previous studies involving heat setting of dilute solutions or suspensions of purified myofibrils or myosins from cod, herring or silver hake (Chan et al., 1992a). The apparent discrepancy between earlier findings in which cross-linking was attributed mainly to noncovalent (hydrophobic) association of MHCs and our results is most probably related to differences in protein concentrations (1–5 mg/mL for model systems; 137.7 mg/mL for surimi in our study). Our results indicated that the textural properties of surimi gels were not due to any single protein-protein interaction and the relative contribution of each type interaction to textural properties are unknown. Our results suggested that the conformational state of myosin molecules appeared to be the most important factor to affect type and extent of chemical interactions in development of the gel network.

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Ms received 5/16/95; revised 7/5/95; accepted 7/27/95.

This work was funded by a Natural Sciences and Engineering Research Council of Canada Strategic Grant awarded to author Dr. Tom A. Gill.

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This research was supported by a grant from the Bonneville Power Administration (Project No. 90-077) and was administered through the Oregon Dept. of Fish & Wildlife and the Dept. of Agricultural & Resource Economics at Oregon State Univ. under the project "Economics, Social and Legal Feasibility of Commercial, Sport, and Bounty Fisheries on Northern Squawfish."

Sorbitol Increases Shelf Life of Fresh Mushrooms Stored in Conventional Packages

S. ROY, R. C. ANANTHESWARAN, and R. B. BEELMAN

ABSTRACT

Trays containing 100g of mushrooms overwrapped with PVC film were held at 12°C. Two 3 mm holes were made on top of the overwrap for ventilation and a Tyvek[R] pouch containing sorbitol was placed at the bottom of the tray before storage. Surface moisture content of mushrooms decreased in the presence of sorbitol. Mushrooms packaged with 10g sorbitol had a constant surface moisture content and those packaged with 15g sorbitol had the best overall color. Principal component analysis of Vis-NIR spectra revealed that surface moisture content affected the scattering of incident light and mushroom color. Lowering of the in-package relative humidity did not affect the maturation rate of mushrooms, but reduced bacterial growth, suggesting that improvement in color was probably due to reduced bacterial activity.

Key Words: mushrooms, moisture-absorber, storage life, sorbitol, relative humidity

INTRODUCTION

OVERWRAPPING MUSHROOMS with plastic film improves their quality as observed by rate of cap opening, color and weight loss (Gormley and MacCanna, 1967; Nichols and Hammond, 1973, 1975). High respiration rates of mushrooms (500 mg CO_2/kg fresh wt/hr at ambient temperature) (Burton and Twyning, 1989) result in a very low O_2 concentration (1–2% O_2 at 18°C) in sealed PVC overwrapped pre-packs (Nichols and Hammond, 1973). To prevent in-package atmospheres from being anaerobic, which can increase risk of *Clostridium botulinum* growth, conventional packages containing mushrooms are perforated at the top with two mm holes according to the 1978 FDA recommendation (Herr, 1991).

Gormley and MacCanna (1967) studied the effect of overwrapping mushrooms with different types of perforated and nonperforated films on quality of mushrooms during storage. They found that water condensation occurred on the underside of the nonperforated film, making the appearance of the mushroom packs unattractive, while there was excessive water loss through the perforated films causing wrinkling and brown patches on mushroom surfaces. They also found that mushrooms whiteness loss was proportional to water loss during storage whether covered or uncovered. However, they did not find a consistent relationship between moisture loss and whiteness loss in mushrooms stored at different temperatures and relative humidities (RH) (MacCanna and Gormley, 1968). Browning or yellowing of sporophore surfaces, bacterial blotch, is caused by *Pseudomonas tolaasii* (Paine, 1919). Water layers persisting on caps support the growth of this bacterium (Barber and Summerfield, 1990).

An optimum surface moisture content for stored mushrooms should be low enough to reduce growth of *Pseudomonas tolaasii*, and high enough to prevent wrinkling and scaling of mushroom surfaces. Our objective was to evaluate the effects of sorbitol as moisture absorber on shelf-life of mushrooms stored in conventional packages at 12°C.

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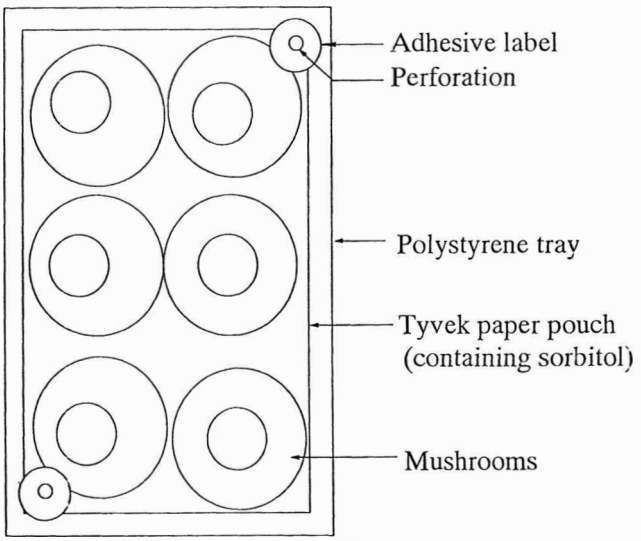


Fig. 1—Diagram of top view of the package.

MATERIALS & METHODS

MUSHROOMS (*Agaricus bisporus*) of U-1 (hybrid off-white) strain were grown on traditional horse manure-based compost at the Mushroom Test and Demonstration Facility (MTDF) at the Pennsylvania State University. Mushrooms from only the first and second flushes (cropping cycle) were used. Freshly harvested mushrooms, free from visual blotches, were transported within 1 hr after harvest and were promptly placed in cold storage at 4°C for ≤4 hr before packaging.

Packaging and storage

Mushrooms were sorted on the basis of size and appearance. Diseased, damaged, open-veiled and extremely large (cap diameter >40 mm) or small mushrooms (cap diameter <25 mm) were discarded. Stems were hand trimmed to a stipe length of 7 ± 2 mm. Acceptable mushrooms were selected at random, and 6 mushrooms were placed into 225g linear polystyrene mushroom trays, so that each tray had 100 ± 5g of mushrooms. Paper pouches were made with Tyvek[R] (Dupont) and different amounts of sorbitol were placed in these pouches. They were sealed with a heat sealer (Foodsaver, Tilia Trust, S. Anselmo, CA) and placed in the tray underneath the mushrooms. Trays were overwrapped with polyvinyl chloride (PVC) film and heat-sealed at the bottom of the package with a hot plate.

A schematic diagram of the package containing mushrooms and the pouch containing sorbitol is shown (Fig. 1). Two 3-mm (in diameter) perforations were made at the top of each package (to promote ventilation), diagonally opposite and equidistant from nearest corners. In order to obtain precise perforations and to avoid accidental tearing of film, holes were punctured through pre-punched self-adhesive labels affixed to the film. The surface area of the film covered by the label (2 cm^2) was negligible compared to the total surface area covering the top of the tray. Packages were stored at 12°C and 80% RH in an incubator (Model 815, Lunair Environmental, Inc., Williamsport, PA).

Measurements of quality characteristics

Six mushrooms were chosen at random to evaluate quality characteristics on day 0. Three trays of mushrooms were selected at random from

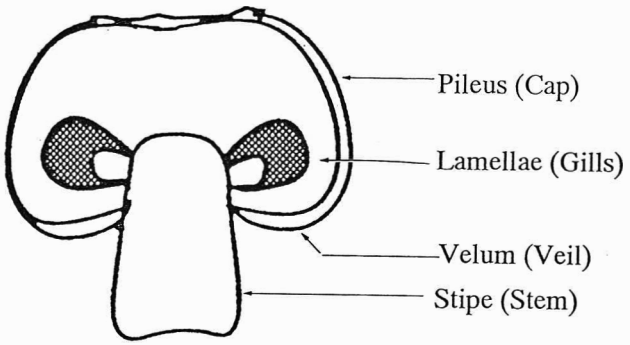


Fig. 2—Cross-section of mushroom.

the low-temperature incubator after 3, 6 and 9 days storage and all mushrooms in each tray were evaluated. Mushrooms were removed from trays and weighed immediately to evaluate fresh weight loss. The sorbitol pouch was also weighed to determine the amount of moisture absorbed. These mushrooms were then used to evaluate maturity, surface moisture, and color of mushrooms.

Maturity index. A diagram of the cross-section of a mushroom is shown (Fig. 2). The maturity index was determined as described by Guthrie (1984). The maturity index was assigned to mushrooms based on extent of cap opening on a 7 point scale (Table 1).

Surface moisture. Since water activity of mushrooms inside the package varies due to respiration and transpiration, surface moisture of mushrooms was used as a quality indicator during storage. Surface moisture was determined using visible/Near Infrared (Vis-NIR) spectroscopy based on the method developed by Roy et al. 1993. The Vis-NIR spectra represent the reflectance energy from the surface of mushrooms and are dependent on RH at the mushroom surface.

This technique was developed only after the first replication of the shelf-life study was completed. Therefore only the second replication was subjected to Vis-NIR spectroscopic analysis for surface moisture content and other principal components. Immediately after measurement of maturity, stipes were trimmed and each mushroom was mounted on the sample holder as described by Roy et al. (1993). The mushroom was then scanned 25 times using a NIRSystem 6500 monochromator (Perstorp Analytical, Silver Springs, MD). To obtain the base line of the spectrum, a ceramic standard was scanned eight times before and after each mushroom. Surface moisture content was determined by using the log(1/Reflectance) spectral data in the calibration equation developed by Roy et al. (1993) and further modified by Roy et al. (1995) using the reference moisture content values (wet basis) determined by convection oven. The calibration equation had a standard error of prediction of surface moisture content of 0.7%.

Principal Component Analysis (PCA). Principal component analysis (PCA) was performed to better understand variations in the Vis-NIR spectra and to relate them to physicochemical parameters. The same calibration file used to predict surface moisture of mushrooms was also used to calculate principal components from the vis-NIR spectral data. Principal component analysis (PCA) describes the multidimensional variation of spectra by means of a small number of uncorrelated variables (Cowe and McNicol, 1985). Since the spectra were obtained using 400 wavelengths (in the range of 600–2200 nm, with a gap of 4 nm), each spectrum was considered as a single point in 400 dimensions, D_1, D_2, . . . , D_{400}. Use of PCA reduces these dimensions to smaller numbers of uncorrelated variables.

A principal component was obtained by rotation of original axes D_1, . . . D_{400} which maximized the variation along that principal component, which became the new axes, P_1 and was termed as the first principal component. The second principal component, P_2, was the axis at right angle to the first (P_1) along which there was a maximum residual variation. The other components were derived at right angles to all preceding principal components, which in turn exhibited the greatest amount of "unexplained" variation in data (Cowe and McNicol, 1985; Cowe et al., 1985). This process was continued until all of variations were partitioned into principal components. Mathematically, principal components are the eigenvectors of the covariance matrix S containing spectral data, and the variances along each of the component axes are the eigenvalues of S. For a set of n sample spectra, if D_{ij} is the deviation of the i-th sample spectra from the mean spectra at the j-th wavelength, then,

$$\mathbf{S} = 1/(n-1)\mathbf{D}^T\mathbf{D} \quad (1)$$

where, D is the matrix containing D_{ij} and D^T is the transpose of D.

Table 1—Classif cation of stages in sporophore development[a]

Stage	Description
1	Veil intact (tight)
2	Veil intact (stretched)
3	Veil partially broken (< half)
4	Veil partially broken (> half)
5	Veil completely broken
6	Cap open, gills well exposed
7	Cap open, gill surface flat

[a] Source: Guthrie (1984)

Separating the principal components resulted in components (P_1, P_2, etc.) being uncorrelated variables, and each successive component had less variation than any of its predecessors.

Principal components were calculated for both log(1/Reflectance) of the spectra and for the standard normal variate transformation (scatter correction) (Barnes et al., 1989) of log (1/Reflectance) of the spectra. P_1, P_2, . . . thus obtained were correlated with the moisture content in order to determine the effects of moisture content on scattering of incident rays. Infrasoft International Software (Shenk and Westerhaus, 1991) was used to obtain the calibration, scan the samples and to estimate the principal components in the spectra.

Color. Surface color of mushrooms was measured using a Minolta Chroma Meter (Model CR-200, Dynamic Electronic Sales, Churchville, PA). Before measurements, the instrument was standardized with a standard white plate (Calibration Plate CR-A43). Three measurements were taken at random locations on the cap of each sporophore. Measured values were compared with the ideal mushroom (target) color values of L = 97, a = −2, and b = 0 (Ajlouni, 1991) using a ΔE parameter as described by the following equation.

$$\Delta E = [\{L-97\}^2+\{a-(-2)\}^2+\{b\}^2]^{1/2} \quad (2)$$

ΔE indicates the degree of overall color change in comparison to color values of an ideal sporophore. Therefore an ideal mushroom would have a ΔE value of zero. ΔE and absolute L values (describes degree of whiteness) were used to describe the color of mushrooms during storage.

Bacterial population

The total plate count was determined as described in Ajlouni (1991). Three packages per treatment were evaluated. All mushrooms in each package were blended with 100 mL of 0.1% peptone diluent in a previously sterilized Oster Blender for 2 min. The homogenate was serially diluted and plated using the spread plate method (0.1 mL per plate) on prepoured Eugon agar plates. The plates were then incubated at 32°C for a period of 48 hr. Three packages treatment were evaluated after 3 and 6 days storage.

Statistical analysis

The experiment was analyzed as a six (treatments) by three (days) factorial model with replications as blocks. Data collected from the evaluation of color, maturity index, surface moisture content and weight loss (or gain) by the mushrooms (or sorbitol) were subjected to analysis of variance (ANOVA). General Linear Model (GLM) Procedure of Statistical Analysis System (SAS Institute, Inc., 1985) was used. The bacterial populations of mushrooms were compared using a t-test (Steel and Torrie, 1980). To control the type I error (the rejection of a true null hypothesis), Bonferroni multiple comparison method with an $\alpha=0.05$ was used to determine differences between treatments within each day.

RESULTS & DISCUSSION

Mushroom weight loss and sorbitol weight gain

Weight loss in mushrooms occurs due to moisture lost to absorber, through perforations and by diffusion through the film, and loss of carbon due to respiration. Patel et al. (1988) observed weight loss in mushrooms even when they were stored at 97% RH. Mushrooms stored without sorbitol lost 3 and 7.5% of their fresh weight after 3 and 9 days storage, respectively (Fig. 3a). The weight loss was within the 8.0% range reported for golden white and 6.7% for off-white strain stored 9 days at 12°C (Schmidt, 1977). Similar weight loss (1.9% and 8.4% after 2 and 10 days, respectively) was also reported by Ajlouni (1991) at 12°C while studying the same U1 hybrid strain. As expected,

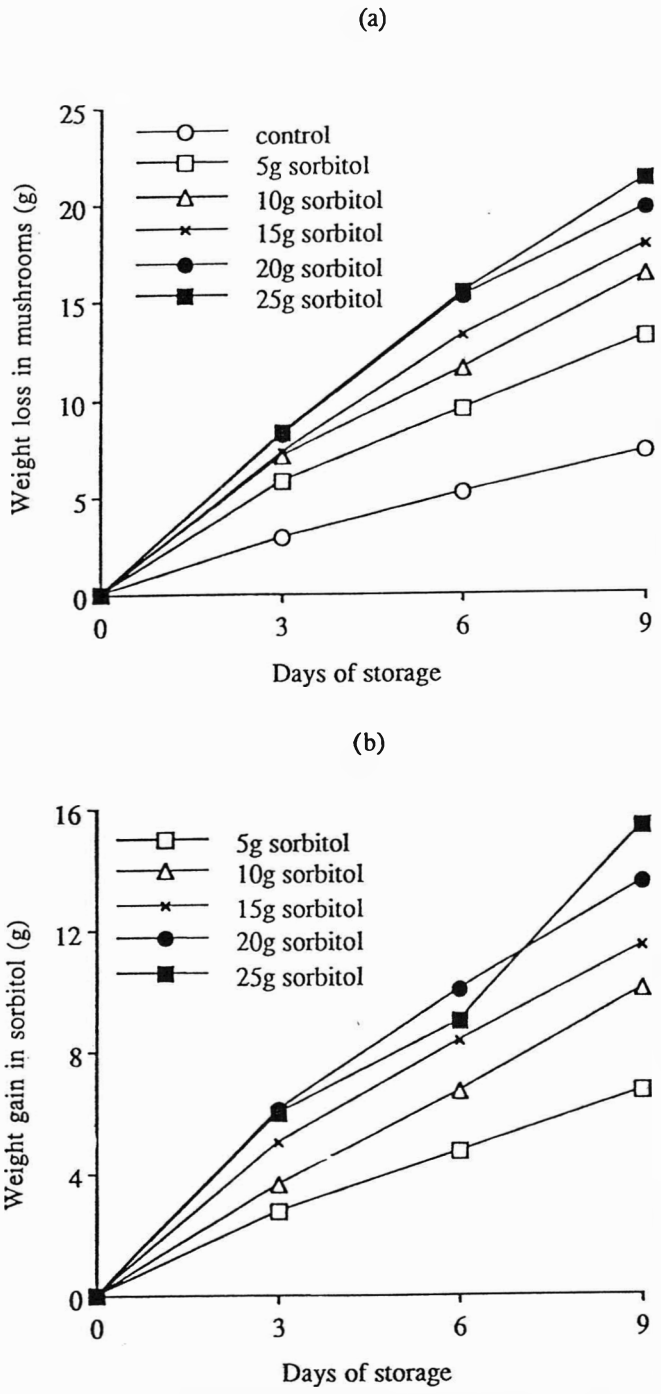


Fig. 3—Effect of sorbitol on (a) weight loss of mushrooms (largest standard error of mean = 1.157), and (b) moisture absorbed by sorbitol (largest standard error of mean = 0.865) during storage in conventional packages at 12°C.

mushrooms stored without sorbitol lost less weight than any other treatment after 9 days storage ($p < 0.05$).

Higher weight loss was noticed with mushrooms packaged with 15g and higher amounts of sorbitol after 3 days storage ($p < 0.05$). Mushrooms with 10g and 5g of sorbitol did not lose more weight than those without sorbitol ($p > 0.05$), until after 6 and 9 days storage respectively. Moisture absorbers such as sorbitol (showing a type III sorption isotherm) adsorb water through hydrogen-bonding to the −OH groups on the surface of sorbitol (Labuza, 1984). Therefore increasing amounts of sorbitol would probably result in higher weight loss in mushrooms.

Sorbitol in 20 and 25g pouches had higher weight gain than that in 5g pouches only after 6 days of storage ($p < 0.05$) (Fig. 3b). This slow initial absorption of moisture by higher amounts of sorbitol could be accounted for in two ways. Moisture absorbers showing type III sorption isotherm are characterized by weak interaction between vapor and a nonporous or macroporous solid (Gregg and Sing, 1982) which has a homogenous surface (Steele, 1974). The occurrence of type III sorption also requires that the heat of adsorption of the first layer (i.e., sorbent-sorbate interaction force) be in the same order of magnitude or smaller than the heat of liquefaction (i.e., sorbate-sorbate attraction) (Shirazi, 1989). Therefore, the uptake of water is small at low vapor pressure and in-package relative humidity (IPRH), due to weak water-solid attraction forces (Shirazi, 1989). As vapor pressure increases beyond a critical point (Labuza, 1984), a considerable increase in water content of the sorbent occurs as a result of the attachment of water molecules to form chains of indefinite length to the first layer. Therefore a slower weight gain would be expected during the initial period due to lower IPRH. Also, since absorption is a surface phenomenon (Labuza, 1984) and since the dimensions of the Tyvek® pouches were the same, all treatments in this study provided similar surface areas during the initial period of storage, resulting in similar weight losses in mushrooms. Once water penetrated into and dissolved sorbitol molecules, new surfaces were exposed for further interactions. Thus only after 9 days storage did pouches containing 25g sorbitol have higher moisture absorption than those containing 10 and 15g sorbitol ($p < 0.05$).

Surface moisture content

Fockens and Meffert (1972) suggested that mushrooms have a surface covered with a thin layer of water or air saturated with water vapor. San Antonio and Flegg (1964) reported a high rate of evaporation of water from the surface of mushrooms during normal growth. Such results imply that mushroom flesh has a very low resistance to flow of water from inside to the surface. There is general consensus among growers and researchers (Kligman and Perry, 1943; Gandy, 1967; Sinden, 1971; Gaze, 1979) that avoiding the persistence of the thin layer of water on the mushroom surface is the best defense against bacterial blotch. Surface moisture was used to monitor "dryness" of the mushroom surface. As expected, mushrooms without sorbitol had higher moisture contents ($p < 0.05$) than those stored with moisture absorbers throughout storage (Fig. 4). Mushrooms stored with 5g sorbitol had higher ($p < 0.05$) surface moisture content than those stored with 25g sorbitol after 6 days storage and those stored with 15 and 20g sorbitol after 9 days of storage. No significant differences ($p > 0.05$) in surface moisture content of mushrooms were observed in packages containing 15, 20 and 25g sorbitol. Mushrooms with 10 and 15g sorbitol had constant surface moisture after 3 days storage ($p > 0.05$). A continuous decrease in surface moisture of mushrooms with 25g sorbitol was observed during storage and the rate of change of surface moisture of these mushrooms were higher ($p < 0.05$) than those with 10g of sorbitol. Rapid decrease in surface moisture with 25g sorbitol might be an indication of overdrying of those mushrooms during storage.

Principal component analysis (PCA)

The spectra of the calibration file used to obtain the "best" equation for predicting moisture content of mushrooms were also used for PCA. The spectra were transformed to the first derivative with a 4 data point gap using a 4 point running-average-smooth (mathematical treatment to obtain calibration equation). The eigenvectors were calculated with and without scatter corrections. These eigenvectors were then used to form principal components. PCA with and without scatter correction was performed to determine effects of surface moisture content on scattering of incident rays. According to Barber and Summerfield (1990), color of a mushroom is dependent on scattering of incident light on mushroom surfaces. When light strikes the surface of a drier mushroom, virtually all of it is reflected to the

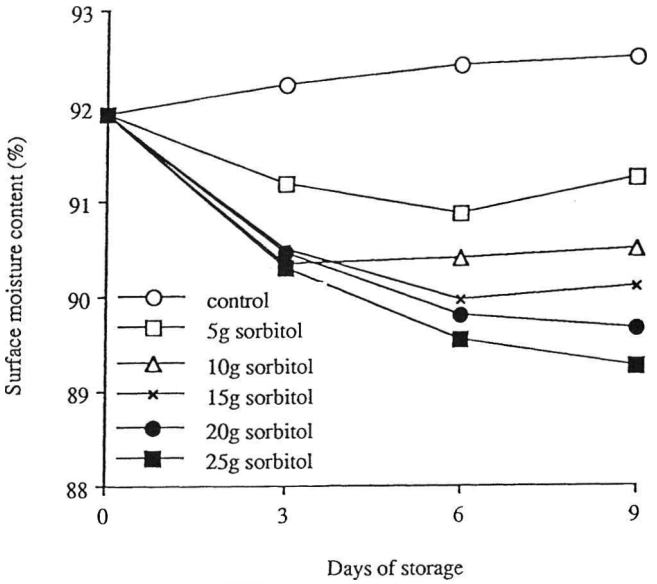


Fig. 4—Effect of sorbitol on surface moisture content of mushrooms (as measured by Vis-NIRS) during storage in conventional packages at 12°C (largest standard error of mean = 0.274).

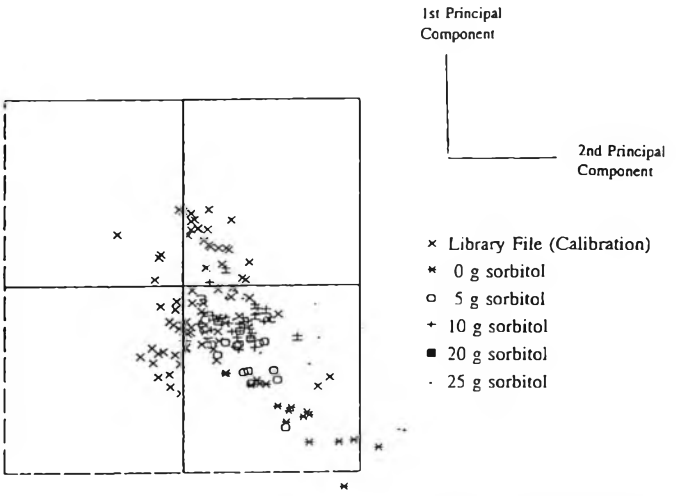


Fig. 5—Effect of sorbitol on the eigenvalues of the principal components of spectra of mushrooms stored for 9 days in conventional packages at 12°C.

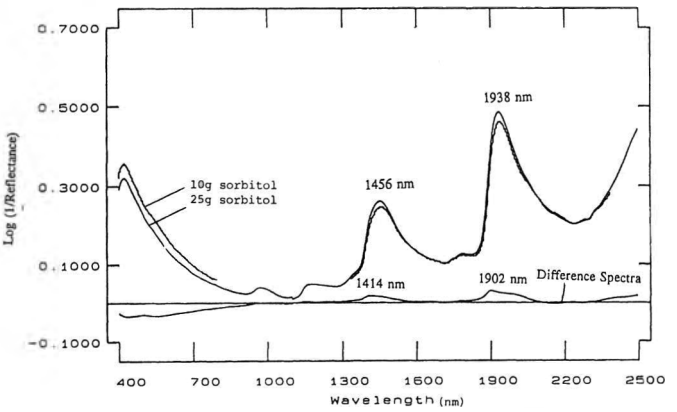


Fig. 6—Log (1/Reflectance) plot of the average spectra and the difference spectra of mushrooms stored for 9 days with 10g and with 25g sorbitol in conventional packages at 12°C.

viewer. In a wet mushroom, water penetrates spaces between mycelial strands and the light is conducted deep into the cap on the watery pathways. Though some light is reflected, more light passes into the tissue and is not returned. This causes mushrooms with a wetter surfaces to appear darker.

The cumulative percentage variation explained by the first three components and their correlation with moisture were compared (Table 2). First component without scatter correction explained 94.82% of the variation of the spectra as opposed to 88.26% with scatter correction. This implied that a portion of variation of the spectra in the first component was due to scattering of the incident rays on the mushroom surface. The increase in correlation coefficient (r) of the first component with the moisture content of the mushrooms from 0.80 to 0.86 with the scatter correction also implied a significant effect of scattering on variation of the spectra. This was in agreement with Cowe and McNicol (1985) who reported that first principal component accounted for the vast majority of the variation and this component could be interpreted as scattering due to particle size. The first principal component also highly correlated with moisture content. This suggests that moisture is important in light scattering at the mushroom surface.

The eigenvalue scores of mushrooms from all of treatments after 9 days storage were plotted using the first two principal components (Fig. 5). As expected, mushrooms stored with sorbitol had higher eigenvalue scores in the first principal component (related to moisture content) than those stored without sorbitol. Mushrooms stored with 10g and higher amounts of sorbitol had similar scores in first component and varied with second. Since very small correlation exists between moisture content and eigenvalue scores of the second principal component (Table 2), our observation indicated a probable physical transformation related to overdrying of surfaces of mushrooms stored with high amounts of sorbitol.

The average of spectra from mushrooms stored with 25 and 10g sorbitol had peaks at 1454 nm and 1938 nm (Fig. 6). However, the difference spectra showed peaks at 1414 nm and 1936 nm. This indicated that moisture resonating at lower wavelengths showed higher differences than average water molecules present in mushrooms. Water molecules resonating at lower wavelengths are associated with fewer hydrogen bonds (free water) (Osborne and Fearn, 1986). Therefore, mushrooms packaged with 10g sorbitol probably had more free water (not associated with the mushroom matrix) than those stored with 25g sorbitol.

Table 2—Percentage of variation of spectra explained by addition of first three principal components and their correlation with moisture

Components	% variation[a]	r (moisture)[b]
A. Without scatter correction		
1	94.82	0.80
2	97.43	0.28
3	97.82	0.27
B. With scatter correction		
1	88.38	0.86
2	93.76	0.15
3	96.98	0.19

[a] Cumulative percentage of variation explained after sequentially adding the component.

[b] Correlation coefficient obtained by correlating the "weights" of the principal component with moisture.

Maturity index

The use of different amounts of sorbitol and variation of IPRH did not have a significant effect ($p > 0.05$) on maturity index (Fig. 7). Similar results were reported by Zoberi (1981) that growth of sporophores before harvest of *Agaricus campestris* remained unaffected by different RH conditions.

There is movement of dry matter from the stipe to the pileus after harvest. Hammond and Nichols (1975) associated this translocation with opening of the cap and breaking of the velum and thus with the rate of maturation. Since transpiration depends on RH of the environment, a higher rate of cap opening (maturity index) would be expected with lower RH if transpiration

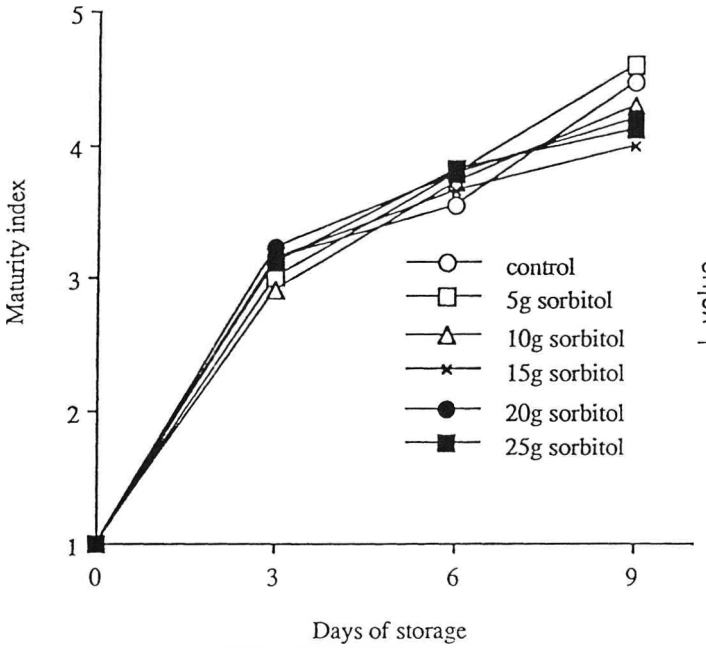
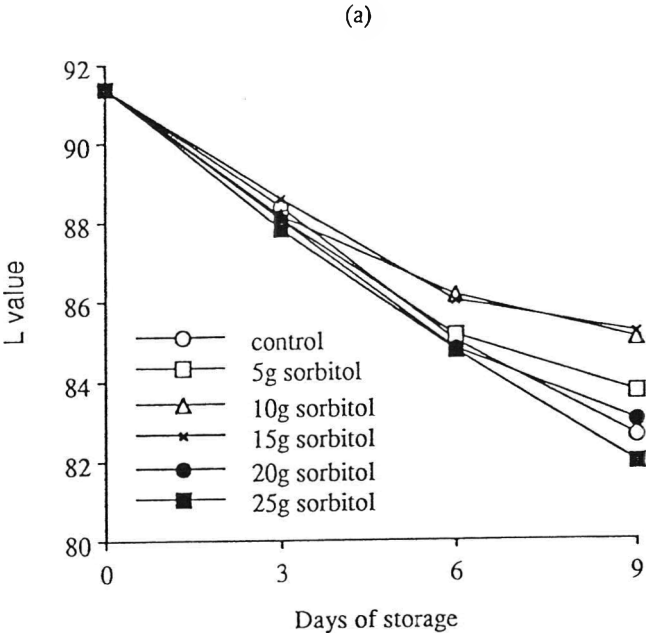


Fig. 7—Effect of sorbitol on the maturity index of mushrooms during storage in conventional packages at 12°C (largest standard error of mean = 0.183).



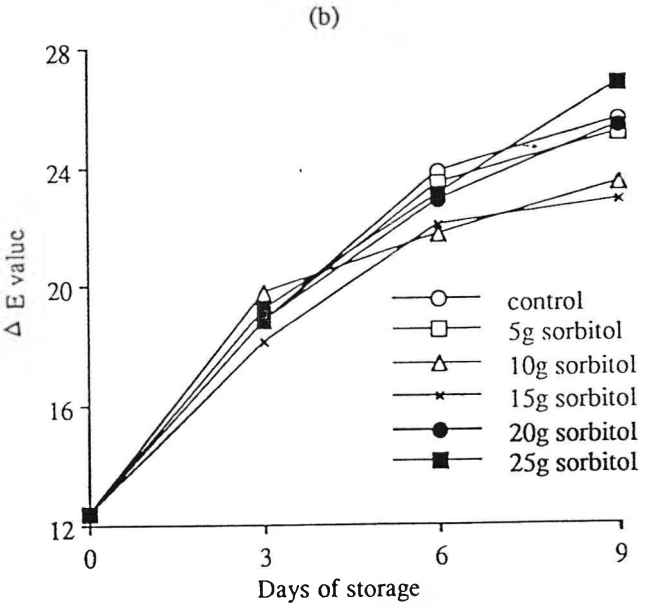


Fig. 8—Effect of sorbitol on color of mushrooms stored in conventional packages at 12°C (a) L value (largest standard error of mean = 0.489), and (b) ΔE value (largest standard error of mean = 0.576).

is the major means of translocation in fungi. Expansion of the cap and extension of the stipe of ink cap mushrooms, *Coprinus atramentarius* and another basidiomycete, *Tricholoma personatum*, were greatly increased by lowering RH from 90 to 30% (Zoberi, 1981). However, in Agaric spore formation, translocation is dependent upon protoplasmic streaming which is less sensitive to evaporational suction (Schutte, 1956). Since the evaporational suction is a secondary phenomenon affecting translocation of mushrooms, the rate of transpiration has very little or no effect on maturity index.

Color

There were significant differences ($p > 0.05$) in color values between various replications of experiments since mushrooms were obtained from 2 different flushes. Bartley et al. (1991) also found that mushrooms from successive flushes were progressively darker prior to harvest and browned at a faster rate during postharvest storage at 12°C. Those stored without sorbitol had lower L values and higher ΔE than those stored with 10 and 15g sorbitol ($p < 0.05$) (Fig. 8). As described besides restricting the growth of *Pseudomonas tolaasii*, a drier mushroom surface looks whiter. With mushrooms, a darker color due to cap dampness is far more common and more of a serious problem than bacterial blotch (Barber and Summerfield 1990).

According to Flegg and Wood (1985) a transpiration rate of <6 mg/cm^2/hr allows a pre-harvested sporophore to grow normally. Above that rate, sporophore surfaces become scaly and discolored, and develop severe surface cracking. Although water flow through interhyphal cavities of mushroom tissue is high (to replenish water lost at the surface), this becomes rate limiting at a very high transpiration rate, and causes overdrying at the surface. Surface moisture was constant when mushrooms were stored with 10g of sorbitol, indicating the rate of water transport from tissue to the surface was the same as the rate of water loss from the surface.

Mushrooms packaged with 20 and 25g sorbitol had lower L values (Fig. 8a) than those with 10 and 15g sorbitol after 9 days storage ($p < 0.05$). However, the same mushrooms had higher ΔE values ($p < 0.05$) than those with 15g sorbitol (Fig. 8b). Since mushrooms stored with 20 and 25g sorbitol had a tendency for continuous drying of the surface during storage (Fig. 2), lower color values associated with those mushrooms were probably due to overdrying. Since mushrooms packaged with 15g sorbitol had the best overall color, conditions attained in these packages were considered to be optimal for the best color of mushrooms during storage.

Bacterial population

Drier mushroom surfaces restrict growth of *Pseudomonas tolaasii* (Barber and Summerfield, 1990). Therefore a lower bacterial count was expected with reduced RH in packages using moisture absorbers. Since mushrooms stored with 15g sorbitol had the best color overall, the total microbial count of those mushrooms was compared with mushrooms stored without sorbitol. Those stored with 15g sorbitol had a lower microbial population ($p < 0.05$) than controls (Table 3).

Table 3—Effect of sorbitol on standard plate count of mushrooms in conventional packages stored at 12°C

Treatment	Total plate count (10^7 CFU/g)	
	After 3 days storage	After 6 days storage
Control (no sorbitol)	3.43[a]	8.47[a]
15 g sorbitol	1.52[b]	3.41[b]

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

Postharvest physiology and pathology must be considered together in evaluating effects of RH on shelf-life of produce. Generally, decay is associated with a high RH. Wong and Preece (1982), Guthrie (1984), Kramer (1986), and Doores et al. (1987) demonstrated that bacterial growth on freshly harvested mushrooms promotes post-harvest deterioration. Doores et al. (1987) and Beelman et al. (1989) suggested that continuous increase in the rate of browning in harvested mushrooms was related to bacterial growth. Since microorganisms are responsible for structural damage that can result in sunken lesions and bruised appearance, bacterial growth can facilitate interactions between tyrosinase and the phenolic substrate, and increase the rate of browning reactions. Studies have also shown that tolaasin, the toxin produced by *Pseudomonas tolaasii*, the causal organism of bacterial blotch disease, was solely responsible for disease symptoms on mushrooms and that it could disrupt fungal cells membranes and thus permit enzyme and substrate contact for browning reactions (Rainey et al. 1991).

CONCLUSIONS

MUSHROOMS packaged in conventional packages with 15g sorbitol had better color (L and ΔE) than those with no sorbitol or with 5, 20 and 25g sorbitol. Mushrooms packaged without sorbitol had higher surface moisture and bacterial activity than those with sorbitol. Mushrooms with 10 and 15g sorbitol had a constant surface moisture after 3 days storage. Those with surface moisture 90% had darker color, probably due to overdrying. A rapid decline of surface moisture of mushrooms to 90% after 6 days storage occurred when they were stored with 25g sorbitol. PCA showed that moisture content affected the scattering of incident rays and thus the color of mushrooms. Graphical analysis of differences in Vis-NIR spectra revealed that sorbitol removed free water from mushroom surfaces. The optimum surface moisture was found to be 90 to 90.5%, which was attained within 3 days storage with 15g sorbitol. Surface moisture is indirectly related to RH at the mushroom surface. Thus packages containing different amounts of mushrooms, with different moisture absorbers (showing type III sorption isotherm) should have increased shelf-life when the surface moisture content is maintained within that range.

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Ms received 8/2/94; revised 2/16/95; accepted 3/17/95.

Chinese Cabbage Midribs and Leaves Physical Changes as Related to Freeze-processing

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ABSTRACT

Differences were investigated in texture, drip and pectic composition of raw or blanched Chinese cabbage midribs and leaves after conventional (F) or program freezing (PF). Softening of tissues and amount of drip were least to greatest: PF-5°C/min < PF-2°C/min < F-35°C < F-20°C, respectively. Freezing-thawing accelerated release of pectin but the freezing rate did not affect pectin release much. Total pectin in raw midribs was less than in raw leaves. The leaves contained more low methoxyl pectin than midribs, but the high methoxyl pectin was almost the same. Cell damage in frozen-thawed midribs and leaves by light- and electron-microscopy appeared extensive.

Key Words: Chinese cabbage, freezing, texture, cell-structure, pectin

INTRODUCTION

TEXTURE IS IMPORTANT in frozen leafy vegetables. It is very difficult to produce frozen Chinese cabbages with good textural quality. After freezing-thawing, firmness decreased, rupture strain increased (Fuchigami et al., 1995a), and consequently, crispness decreased. Slow freezing resulted in formation of large crystals and irreversible damage to cell structure and texture (Reeve, 1970, Goodenough and Atkin, 1981). Thus, the rate of freezing was critical to tissue damage (Rahman et al., 1971). The optimum freezing rate of carrots has been established as −5°C/min, using a programmable freezer and based on texture and histological structure. (Fuchigami et al., 1994, 1995b). Chinese cabbage leaves cracked when frozen rapidly with liquid nitrogen (LN_2). Our main objective was to determine the optimum rate of freezing Chinese cabbages using a program freezer and conventional freezer.

Differences in texture and pectic composition of raw, cooked and blanched-frozen-then-thawed Chinese cabbage midribs and leaf positions (10, 20, 30, 40, or 50th midribs from outside) have been shown (Fuchigami et al., 1995a). However, similar research relating to leaves has not been reported. A secondary objective was to clarify differences of texture, pectic composition, and histological structure between midribs and leaves of raw, cooked, and frozen-thawed Chinese cabbages.

MATERIALS & METHODS

Sample preparation

The 20 ~ 30th leaves taken from the outside of Chinese cabbage (*Brassica Pekinensis Rupr.*, Satokaze) harvested in June in Nagano, Japan, were used. They were separated into two parts: midribs (white parts) and leaves (light green parts). The middle segments of midribs were cut into 2 cm × 2 cm pieces. The leaves were cut into 5 cm × 5 cm pieces. Samples (4g) were used from each lot. Two replications were done in each experiment.

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Cooking, freezing and thawing

Raw or blanched samples (1 min in boiling water) were with LN_2 (freezing rate: −5°C/min or −2°C/min, final freezing temperature: −20°C) using a program freezer (Cooling Mater CM-Mark II, Taiyo Sanso Co. Ltd., Tokyo), or were frozen at −35°C or −20°C using a conventional freezer (Fuchigami et al., 1994). Samples were thawed 40 min at 20°C or cooked 1 min in boiling water.

Measurement of texture

Firmness of raw, cooked, and frozen-thawed Chinese cabbages was measured using a rheometer (NRM-2002J, Fudoh Kogyo Co. Ltd., Tokyo, interpenetrated type cylindrical plunger; 2 mm in diameter). The epidermis of midribs was removed after thawing, and the firmness of mesophyll (ca. 4 mm thickness) was measured. To measure under the same conditions (4 mm thickness) as midribs, 5 pieces of leaves (avoiding veins) were placed on top of each other, after which firmness was measured at 20°C.

Extraction and determination of pectic substances

Fractionation/chromatography method. Pectic substances in raw, cooked or frozen-thawed Chinese cabbages were extracted successively by four reagents: 0.01N HCl (pH 2.0, at 35°C, 24 hr × 5); 0.1M sodium acetate buffer (pH 4.0, at 35°C, 24 hr × 3); 2% sodium hexametaphosphate (pH 4.0, at 90°C, 3.5 hr × 3); and 0.05N HCl (at 90°C, 4 hr × 3) solutions (Fuchigami and Okamoto, 1984, Fuchigami, 1987). These extracts were designated as PA, PB, PC, and PD (Pectin A ~ D). DEAE-cellulose column chromatography of PA and PB was performed using the method reported previously (Fuchigami, 1987). Galacturonic acid and neutral sugars were determined by the carbazole method (Galambos, 1969) and the phenolsulfuric acid method (Dubois et al., 1956), respectively. The degree of esterification (DE) of pectic substances was measured by a gas chromatographic procedure (Bartolome and Hoff, 1972).

Differential extraction method. Pectic substances were successively extracted from AIS (alcohol insoluble solids) using three reagents: distilled water (at 20°C, 24 hr); 0.4% sodium hexametaphosphate (at 20°C, 24 hr); and 0.05N HCl (at 85°C, 4 hr) (Bettelheim and Sterling, 1955, Miura and Mizuta, 1962). Reagents were exchanged one time during extraction and water-soluble pectin (WSP) and total pectin were determined.

Microscopic observations

Changes in the histological structure of raw, raw-frozen (−35°C)-thawed (20°C), boiled (2 min), or blanched (1 min)-frozen (−35°C)-cooked (1 min) midribs and leaves were observed using a light microscope (LM), a transmission electron microscope (TEM) and a scanning microscope (S-4500, Hitachi Co. Ltd., Tokyo) with cryo-system (cryo-SEM) as reported (Fuchigami et al., 1994, 1995b).

RESULTS & DISCUSSION

Effect of freezing rate on firmness, drip, and pectic substances of midribs

Firmness, drip and pectic substances in midrib samples from differential extraction from AIS were compared (Table 1). When raw midribs were frozen by PF, then thawed at 20°C, firmness was maintained, but midribs frozen by F were softer. Also, midribs thawed at 100°C were softer than those thawed at 20°C. Although cooking time was the same, raw midribs thawed at 100°C were firmer than blanched midribs thawed at 20°C.

Table 1—Effect of freezing rate on firmness, drip, and galacturonic acid remaining in tissues of raw or blanched midribs

	Freezing method	Firmness (× 10^3N/m^2) Thawed at 20°C, 40 min	Firmness (× 10^3N/m^2) Cooked 100°C, 1 min	Drip (%) Thawed 20°C	Drip (%) Cooked 100°C	WSP[a] T-pectin[b]	WSP[a] T-pectin[b] Thawed 20°C	WSP[a] T-pectin[b]	WSP[a] T-pectin[b] Cooked 100°C
	Nonfrozen	958 ± 94	956 ± 63			3[a]	272[b]	5[a]	242[b]
	Program Freezer −5°C/min	959 ± 75	834 ± 154	17.1	19 6	3	233	5	223
Raw	−2°C/min	946 ± 125$^-$	840 ± 140$^-$	17.6	32 1	3	220	4	223
	Freezer −35°C	818 ± 72***	818 ± 140$^-$	26.3	40 3	3	213	4	209
	−20°C	803 ± 61***	620 ± 76***	26.5	44.0	3	209	4	204
	Nonfrozen	956 ± 63	832 ± 119			5	242	6	228
Blanched	Program Freezer −5°C/min	723 ± 64	670 ± 121	31.4	44.0	5	233	6	228
for	−2°C/min	713 ± 97**	557 ± 77**	33.1	45.3	5	223	5	229
1 min	Freezer −35°C	711 ± 124**	504 ± 98***	35.3	48.7	6	217	6	223
	−20°C	643 ± 86**	495 ± 73***	36.3	46.0	5	213	4	206

[a] Water-soluble pectin remaining in tissues (mg/100g).
[b] Total pectin remaining in tissues (mg/100g).
$^-$ $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: significant differences by T-test (pair to program freezer −5°C/min).

Table 2—Effect of freezing rate on firmness, drip, and galacturonic acid remaining in tissues of raw or blanched leaves

	Freezing method	Firmness (× 10^3N/m^2) Thawed at 20°C, 40 min	Firmness (× 10^3N/m^2) Cooked 100°C, 1 min	Drip (%) Thawed 20°C	Drip (%) Cooked 100°C	WSP[a] T-pectin[b]	WSP[a] T-pectin[b] Thawed 20°C	WSP[a] T-pectin[b]	WSP[a] T-pectin[b] Cooked 100°C
	Nonfrozen	857 ± 253	1057 ± 156			29[a]	647[b]	15[a]	614[b]
	Program Freezer −5°C/min	1099 ± 168	1252 ± 136	7.0	15 8	11	521	7	518
Raw	−2°C/min	1105 ± 165$^-$	1071 ± 257*	5.3	18 8	11	518	6	527
	Freezer −35°C	894 ± 198**	735 ± 28***	13.1	30.0	10	506	8	513
	−20°C	864 ± 249**	740 ± 68***	17.5	31.0	10	502	9	512
	Nonfrozen	1057 ± 156	1010 ± 170			15	614	32	607
Blanched	Program Freezer −5°C/min	926 ± 205	781 ± 196	6.0	25.8	7	517	10	503
for	−2°C/min	819 ± 271$^-$	801 ± 211$^-$	9.1	32.5	5	506	9	502
1 min	Freezer −35°C	841 ± 152$^-$	776 ± 140$^-$	8.9	33.8	12	521	4	515
	−20°C	786 ± 127*	770 ± 123$^-$	9.7	35.8	11	513	12	509

$^-$,*,**,***,[a], and [b]: See Table 1.

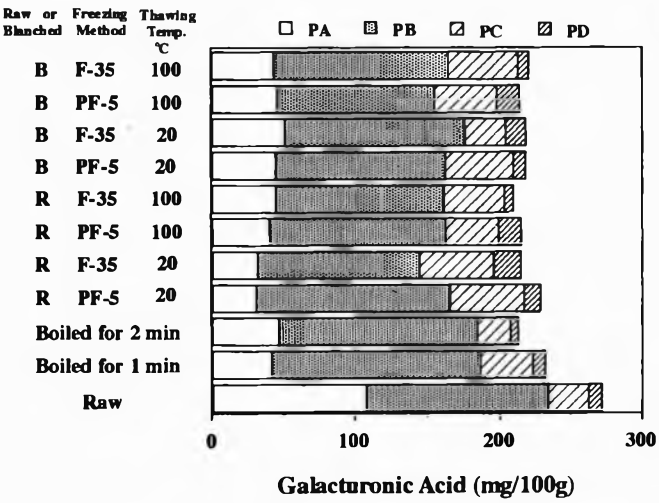

Fig. 1—Changes in pectic composition of midribs during freezing-thawing. PF-5: frozen at −5°C/min by a program freezer, F-35: frozen at −35°C by a conventional freezer. PA: 0.01N HCl-soluble pectin, PB: acetate buffer-soluble pectin, PC: sodium hexametaphosphate-soluble pectin, PD: 0.05N HCl-soluble pectin.

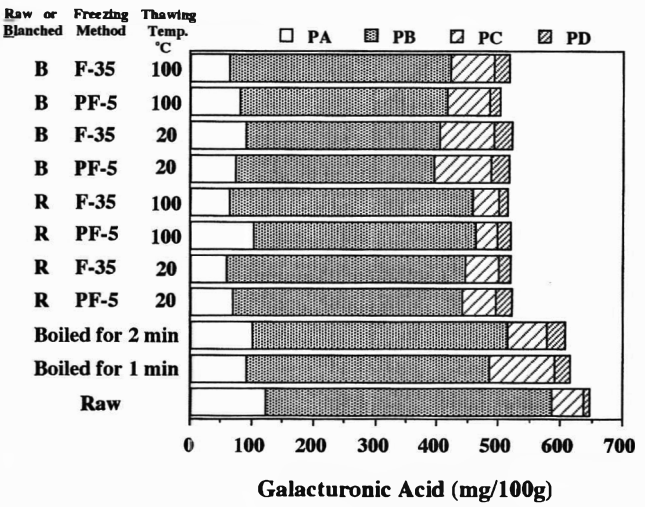

Fig. 2—Changes in pectic composition of leaves during freezing-thawing. Symbols: See Fig. 1.

Blanched-frozen-then-cooked midribs were softest due to maceration in cooking. Regarding rate of freezing, the firmness of frozen-thawed midribs was: PF-5°C/min > PF-2°C/min > F-35°C > F-20°C.

Amount of drip was greatly affected by freezing rate, especially the raw midribs, with drip losses from: PF-5°C/min < PF-2°C/min < F-35°C < F-20°C. Drip from blanched midribs was greater than that from raw midribs.

Water-soluble pectin levels in raw midribs were low, and increased only slightly after blanching and freezing-thawing. Total pectin in midribs decreased during blanching and freezing-thawing. When a quick freezing rate was used, pectin remaining in tissues was slightly greater except for those blanched-frozen-cooked.

Effect of freezing rate on firmness, drip, and pectic substances of frozen leaves

When raw samples were cooked 2 min or frozen-then-thawed at 20°C, firmness of leaves increased (Table 2). Conversely, the mesophyll of midribs, from which the epidermis was peeled, softened. The epidermis of leaves was not peeled off, and five pieces of leaves were placed on top of each other when firmness was measured. Therefore, epidermis elasticity could have affected the firmness of cooked or frozen-thawed leaves. The firmness of raw leaves frozen by PF, then thawed at 20°C was greater than that of leaves frozen by F ($p < 0.01$). When frozen leaves were cooked, raw leaves were firmer ($p < 0.001$) as the freezing rate was increased. However, firmness of blanched leaves was not affected by freezing rate.

The drip from leaves was less after quick-freezing and the drip was less than from midribs. The cause appeared to be the

Table 3—Change in degree of esterification (%) of pectic substances in Chinese cabbages during freezing-thawing

		Nonfrozen			Frozen-then-thawed (20°C)				Frozen-then-cooked			
		Raw	Boiled		Raw		Blanched		Raw		Blanched	
	Pectin		1 min	2 min	PF-5[a]	F-35[b]	PF-5[a]	F-35[b]	PF-5[a]	F-35[b]	PF-5[a]	F-35[b]
Midribs	PA[c]	74.1	69.4	69.7	68.6	68.1	78.4	74.2	70.3	62.5	74.0	67.8
	PB[d]	50.2	45.6	41.0	34.6	35.7	51.8	43.3	43.1	40.3	45.7	42.8
Leaves	PA[c]	69.0	63.4	61.7	56.4	49.1	58.3	61.1	56.9	53.7	51.3	59.7
	PB[d]	52.4	52.6	52.6	34.5	41.7	46.1	48.9	38.6	37.9	39.0	44.0

[a] Frozen using a program freezer (rate: −5°C/min).
[b] Frozen at −35°C using a conventional freezer.
[c] 0.01N HCl-soluble pectin.
[d] Acetate buffer-soluble pectin.

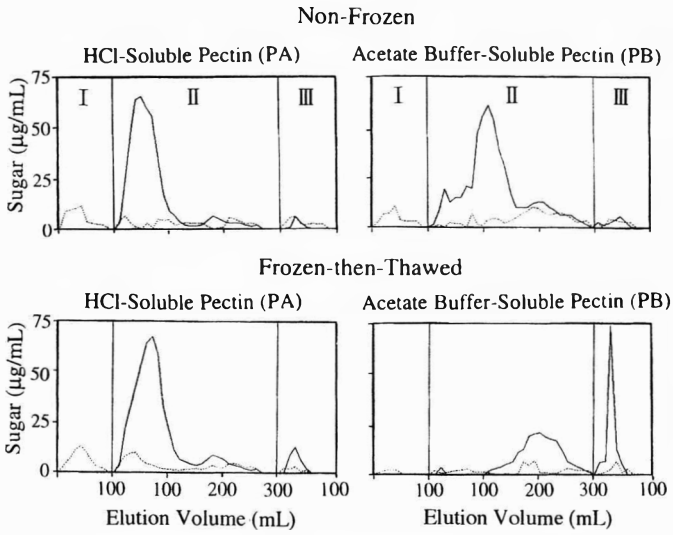

Fig. 3—Changes in DEAE-cellulose column chromatogram of HCl-soluble pectin (PA) and acetate buffer-soluble pectin (PB) in midribs during freezing-thawing. ——— galacturonic acid, - - - neutral sugar. I 0.02M acetate buffer, II gradient elution (0.1M → 1M acetate buffer), III 0.1N NaOH.

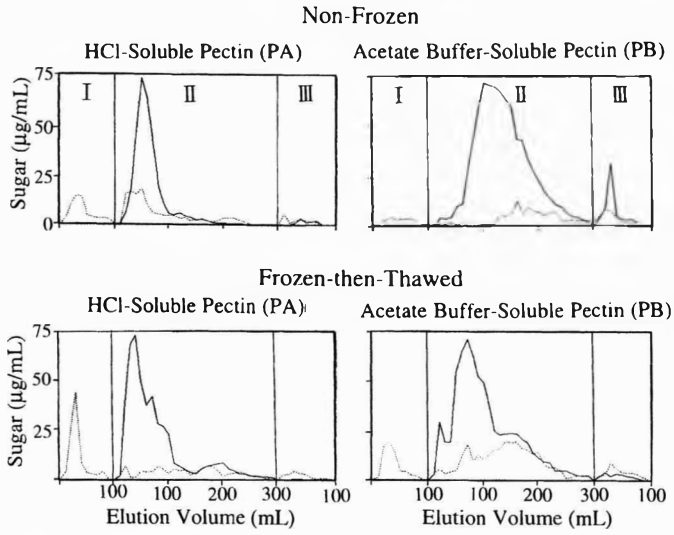

Fig. 4—Changes in DEAE-cellulose column chromatogram of HCl-soluble pectin (PA) and acetate buffer-soluble pectin (PB) in leaves during freezing-thawing. Symbols: See Fig. 3.

smaller cell size, higher pectin and lower moisture of leaves than that of midribs. Moisture in raw midribs was 97.0%, compared to 94.6% in leaves.

Pectin in leaves was greater than that in midribs. The percentage of WSP/total pectin in leaves was comparatively small. After freezing-thawing, the pectin remaining in leaves decreased, but differences in amounts of pectin remaining in the leaves due to freezing rats were not apparent.

Effect of freezing rate on pectic composition of midribs and leaves

Changes in the pectic composition successively extracted by fractionation/chromatography from midribs and leaves after blanching, freezing and thawing were compared (Fig. 1 and Fig. 2). Total pectic substances in midribs was less than that in leaves. Leaves contained more PB (low methoxyl pectin) than midribs but the amount of PA (high methoxyl pectin) was almost the same. Release of pectin from midribs during cooking was greater than that from leaves, and PA in midribs decreased more than PA in leaves. Freezing-thawing accelerated release of pectin but freezing rate did not greatly affect pectin release.

The DE of pectic substances from midribs and leaves was compared (Table 3). PA in raw midribs and leaves was highly esterified, 74.1% and 69.0%, respectively, while the DE of PB was lower (50.2% and 52.4%). The DE of PA in raw midribs was greater than that in leaves thus pectic release from the midribs may have been greater than that from leaves during cooking due to transelimination. Freezing-thawing decreased DE of PA and PB.

Changes in DEAE-cellulose column chromatograms of PA and PB from raw midribs and leaves after freezing (PF-5°C/min)-thawing (at 20°C) were compared (Fig. 3 and Fig. 4). There was no difference between raw midribs and leaves in PA and almost all of it was eluted early in fraction II, confirming that DE of PA in Chinese cabbage was high. Almost all PB was eluted in fraction II also, but it eluted later than PA, indicating a lower DE than that of PA.

In both midribs and leaves after freezing and thawing, PA (eluted later in fraction II) increased slightly, but the chromatogram of PB in midribs showed typically low methoxyl pectin (eluted in fraction III or later in fraction II). These results suggest that freezing then thawing at 20°C may have decreased the DE of the pectin due to pectin methylesterase.

LM, TEM and cryo-SEM observations

Microscopic observations of raw (1), raw-frozen-thawed (2), cooked (3), and blanched-frozen-cooked (4) midribs by LM (a, b), TEM (c) and cryo-SEM (d) were compared (Fig. 5). The vascular system was observed by LM (embedded in paraffin, then stained with safranine-lissamine green) (a-1~4). Cell damage was observed when midribs were frozen then thawed (a-2, a-4), and damage around the vascular system was especially great. The central part of the vascular system was thickest in the midribs, therefore cell damage increased.

Cells of parenchyma were observed by LM (embedded in Epok 812 then stained with toluidine blue) (b1 ~ 4) and TEM (c1 ~ 4). Cells of raw midribs had large vacuoles (b-1), and cytoplasm was pressed against the walls by turgor pressure of the vacuoles (c-1) which explains why raw Chinese cabbages were crisp. The middle lamella, rich with pectin, stained darker than the primary wall (c-1). Microfibrils in primary walls of raw

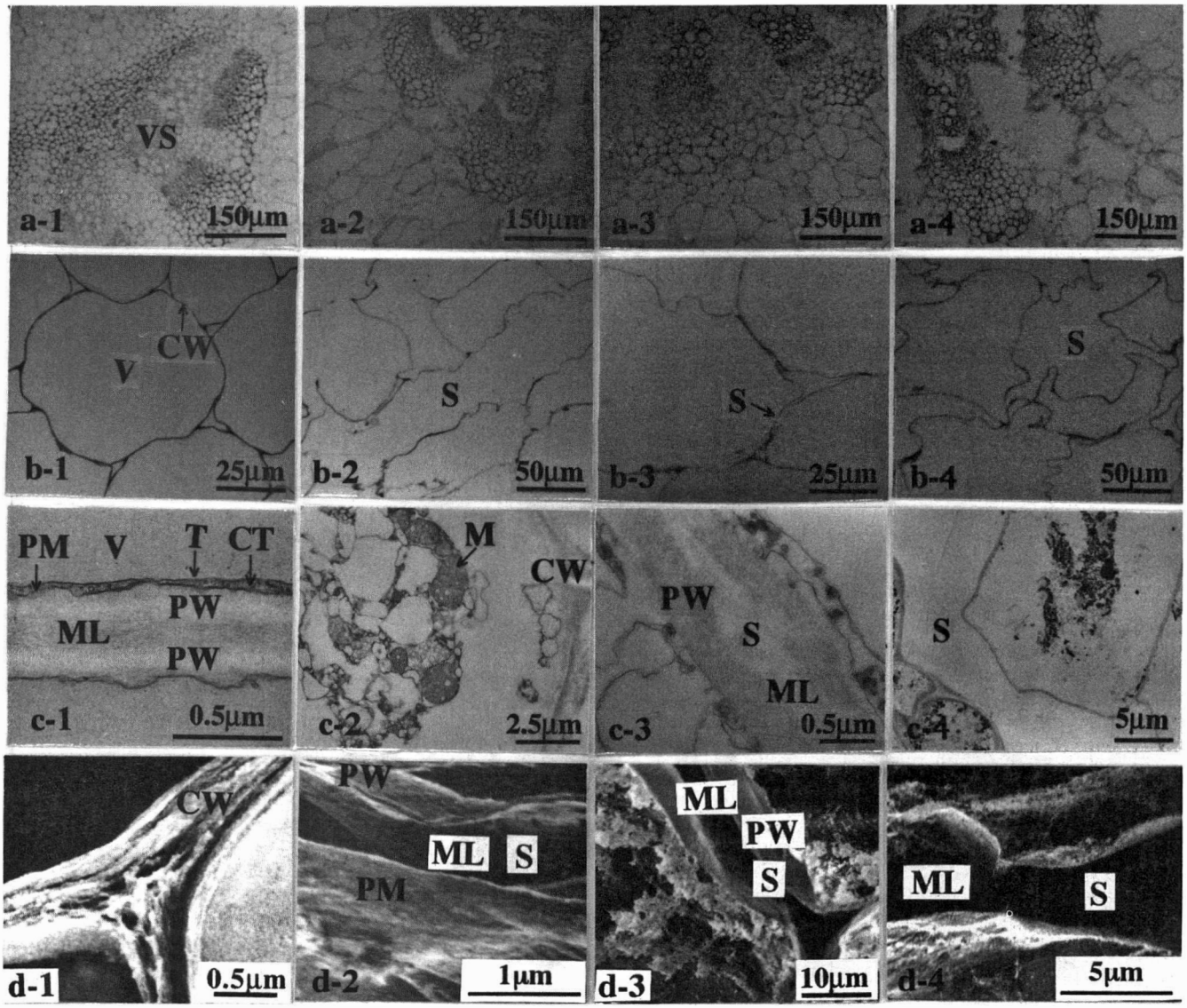

Fig. 5—Light and electron micrographs showing the vascular system and parenchyma of raw, cooked or frozen-thawed midribs. (1) raw; (2) raw-frozen-thawed at 20°C; (3) boiled for 2 min; (4) blanched-frozen-cooked; (a) LM (paraffin); (b) LM (epon); (c) TEM; (d) cryo-SEM. CT: cytoplasm; CW: cell wall; M: mitochondria; ML: middle lamella; PM: plasma membrane; PW: primary wall; S: separated region of the cell wall; T: tonoplast; V; vacuole; VS: vascular system.

midribs were observed by cryo-SEM (d-1). The cell size of midribs was larger than that of leaves which may explain why freezing tolerance of midribs was less than that of leaves.

When raw midribs were frozen then thawed at 20°C, cell separation of parenchyma occurred (a-2, b-2, d-2). The rupture of tonoplasts and plasma membranes would have decreased turgor and increased the amount of drip. Consequently, cytoplasm from the cells was spread throughout (c-2), causing the observed wilting.

When midribs were boiled for 2 min, observed maceration of the middle lamella suggested pectic degradation (b-3, c-3, d-3). Denatured cytoplasm was observed along the cell walls (c-3), but nothing was observed in the center of the cells (b-3). When blanched midribs were frozen then cooked, cell damage was greatest (a ~ d-4). Cells were wilted and denatured. Dark stained cytoplasm was spread throughout the cells (c-4), and large cell separations were observed (a ~ d-4).

Histological changes of leaves were observed (Fig. 6). Cell size of leaves was smaller than that of midribs (b-1, Fig. 5 and b-1, Fig. 6) obvious. Raw leaves had mesophyll of spongy tissues sandwiched between the epidermis (one layer). Spongy tissues had many intercellular spaces and air spaces (b-1). These were probably the cause of less drip from frozen-thawed leaves. Cytoplasm was observed in cells of raw mesophyll which was difficult from cells of midribs. The volume of vacuoles was small, and nuclei and mitochondria were observed (c-1). The greater amount of pectin and less moisture in leaves than in midribs were probably due to the smaller cell sizes in the leaves. When raw leaves were frozen then thawed at 20°C, damage to the nucleus and chloroplast was observed (c-2).

When leaves were boiled, cytoplasm was denatured and stained dark. The tonoplasts and plasma membranes ruptured, and maceration of the middle lamella was observed (b-3, c-3). After blanching-freezing-thawing, cell damage increased (b-4, c-4).

When raw and blanched midribs were frozen by PF, they were firmer than those frozen by F. However, cell damage was observed even after freezing at −5°C/min. The cause of differences in freezing tolerance between midribs and leaves could have been that midribs had larger cell size and lesser pectin. Changes in the amount of drip were more related to texture of midribs, than were changes in pectic composition or histological structure.

CONCLUSIONS

CELL SIZE OF LEAVES was smaller and pectin content (especially low pectin) in leaves was greater than those of midribs. Release

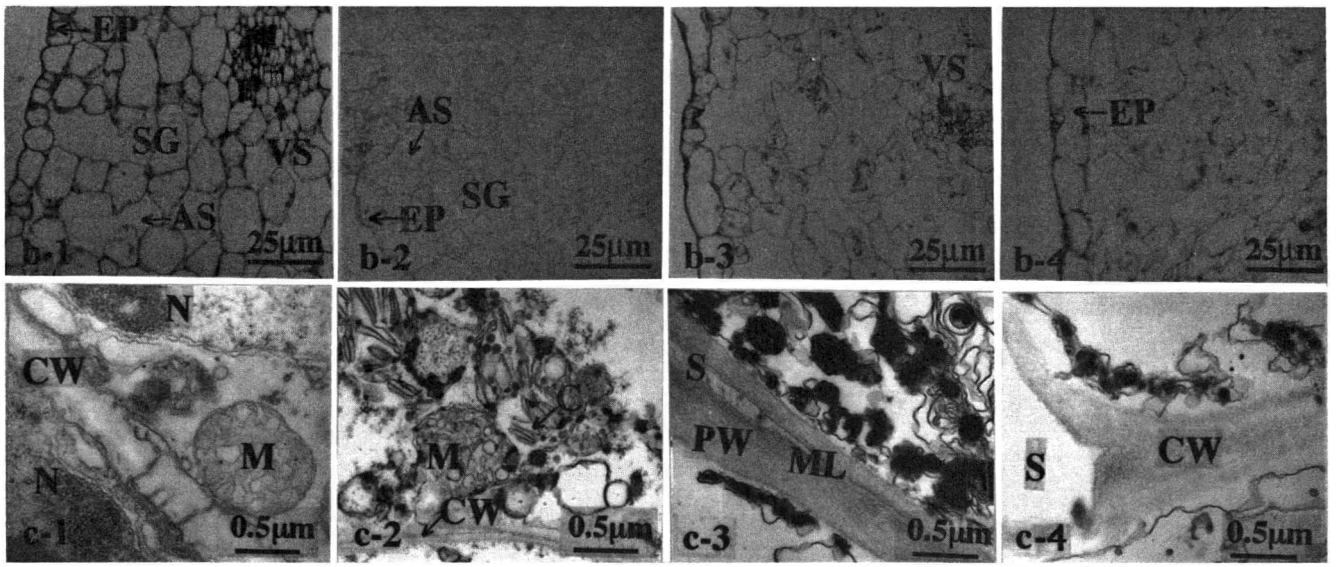

Fig. 6—Light and electron micrographs showing parenchyma of raw, cooked or frozen-thawed leaves. Symbols: See Fig. 5. AS: air space; C: chloroplast; EP: epidermis; N: nucleus; SG: spongy tissue.

of pectin from leaves during cooking was less than that from midribs. Softening of tissues and amount of drip increased when freezing rate was slow. Freezing-thawing accelerated release of pectin but the freezing rate did not greatly affect pectin release. The drip from leaves was less than from midribs. Cell damage in frozen-thawed midribs and leaves by light- and electron microscopy was extensive even after quick freezing. The optimum rate of freezing was −5°C/min with the lowest amount of drip.

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Ms received 2/17/95; revised 6/16/95; accepted 7/18/95.

We thank Mrs. A. Emi for technical assistance. A part of this work was supported by the Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science and Culture in Japan.

Biochemical Changes and Sensory Quality of Shredded and MA-Packaged Iceberg Lettuce

HANNE HEIMDAL, BIRKA FALK KÜHN, LEIF POLL, and LONE MELCHIOR LARSEN

ABSTRACT

Cultivars of shredded iceberg lettuce (*Lactuca sativa* L.) 'Saladin', 'Santis', 'Telda' and 'Roxette' responded in a parallel way to four modified atmosphere (MA) packaging treatments. Moderate vacuum packaging (mvp) in 80 µm polyethylene (PE) inhibited enzymatic browning over 10-day storage at 5°C. When lettuce was packaged in 80% O_2, 20% CO_2 (80/20), more browning occured in SL3-bags (59 µm multilayer coextruded film) than in PE-bags. Polyphenol oxidase activity declined during storage in all 4 atmospheres, with lowest activity in PE-80/20. Endogenous ascorbic acid (AA) did not act as an efficient antioxidant in delaying browning. Total carbohydrate content (TC) declined slightly during storage. Tristimulus color measurements correlated highly with visual ratings.

Key Words: *Lactuca sativa*, enzymatic browning, polyphenoloxidase, ascorbic acid, carbohydrates

INTRODUCTION

INCREASING CONSUMPTION of fast food and prepared salads demands better ways to minimize decreasing quality during processing and storage. Enzymatic browning of processed lettuce is a major problem because visual quality is very important. Polyphenoloxidase (PPO, EC 1.10.3.1) is responsible for browning after tissue injury (Sharples et al., 1963). When lettuce is processed, the tissue is wounded and an increase in soluble phenolic content with no difference in PPO activity has been reported (Ke and Saltveit, 1989c).

Shelf-life of shredded lettuce has been prolonged by using MA-packaging (Ballantyne et al., 1988; Day, 1989; Eskin, 1989; McDonald and Risse, 1990; Bolin and Huxsoll, 1991). The influence of atmospheric composition on biochemical metabolism in lettuce leaf segments was investigated in various controlled atmosphere (CA) experiments (Siriphanic and Kader, 1985, 1986; Ke and Saltveit, 1989a, b, c; Couture et al., 1993) and reviewed by Burton (1974).

When lettuce is harvested, the only energy sources in tissue are free carbohydrates and amino acids. Degradation rate of carbohydrates during storage can indicate the level of metabolism in shredded lettuce. Bolin and Huxsoll (1991) reported no change in sugar content but a decreasing content of soluble solids (Brix) in shredded iceberg lettuce during storage. However, they did not report on various cultivars, the content of ascorbic acid (AA) or PPO activity.

Browning caused by PPO may be delayed as long as AA is oxidized and quinones reduced back to the phenolic level (Walter and Purcell, 1980). Initial AA levels in different cultivars could therefore influence enzymatic browning. No simple relationship between browning, phenolic content and/or PPO was found for fruits and vegetables (Matheis 1983). One reason could be inadequate methods for evaluation of browning (Nicolas et al., 1994). Another could be the rather complex and not fully understood interactions between phenolic substrates, cosubstrates, antioxidants, enzymatic activities and chemical polymerization which results in the browning reactions. Various cultivars of lettuce did not have identical browning activities when shredded and stored under CA (Ke and Saltveit, 1989d; Couture et al., 1993).

Our objective was to correlate the degree of browning in four shredded lettuce cultivars ('Saladin', 'Santis', 'Telda' and 'Roxette') commercially grown in Denmark and stored under different MA conditions with the various activities of the tissue, i.e., enzymatic (PPO), antioxidative (AA) and energy source (total carbohydrates, TC).

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MATERIALS & METHODS

Samples

Cultivars of iceberg lettuce (*Lactuca sativa* L.): 'Santis', 'Telda', 'Saladin' and 'Roxette', commercially grown in Denmark in 1993, were used. These are main cultivars in Denmark. 'Santis' and 'Saladin' have low browning activity (Kjeldsen and Johansen, 1992), while that of the two other cultivars was unknown. Lettuce heads were harvested at optimal maturity for each cultivar and then immediately processed. The lettuce heads were trimmed, halved, stalks removed, and shredded with a RG-400-Vegetable cutter (Hälde, Sweden) into 6 mm pieces. These were washed in 0°C water, and centrifuged for 45 sec at 800 rpm with a kitchen centrifuge.

Shredded lettuce was packaged with a vacuum chamber system (C10H, Webomatic, Germany) in 200g bags. Two packaging materials were chosen because they are used by commercial processors. An 80 µm polyethylene (PE) film and a 59 µm multilayer coextruded film (Cryovac, SL3) were used with oxygen permeabilities at 5°C of 600 and 1205 $cm^3\ day^{-1}\ m^{-2}\ bar^{-1}$, respectively (Andersen, 1994). Bags were stored at 5°C for 10 days except for a few samples stored for sensory analysis up to 17 days.

Four treatments and codes were used: (1) SL3-atm: SL3-bags, atmospheric air; (2) PE-mvp: PE-bags, moderate vacuum packaging (pressure reduced by evacuating to a pressure of 0.45 atm.); (3) SL3-80/20: SL3-bags, 80% O_2, 20% CO_2; and (4) PE-80/20: PE-bags, 80% O_2, 20% CO_2. The gas mixture with high O_2 and high CO_2 concentrations was used because it is used commercially and a preliminary experiment showed no browning after 10 days storage.

Gas measurements

Composition of atmosphere (O_2, CO_2, and N_2) in packages was measured during storage with a 82-12 (VLD) gas chromatograph (Mikrolab, Denmark) equipped with a thermal conductivity detector and a 60/80 Molsieve 5Å, 100/120 Chromosorb 102 twin column (Mikrolab, Denmark). Percentage composition was calculated by using external standards. For analysis, 1 mL was taken from the bags through a rubber membrane (Mikrolab, Denmark) with a plastic syringe. The average was calculated from analysis of three samples on 5 bags from each experimental level.

Color measurement

Degree of browning was measured on a D25-PC2 (HunterLab) tristimulus colorimeter with port diameter 54 mm and reported as L, a and b values; L=light/dark, a=green/red and b=yellow/blue. Three derived functions were computed from the L,a,b readings as follows:

Hue angle: $\tan^{-1}(b/a)$

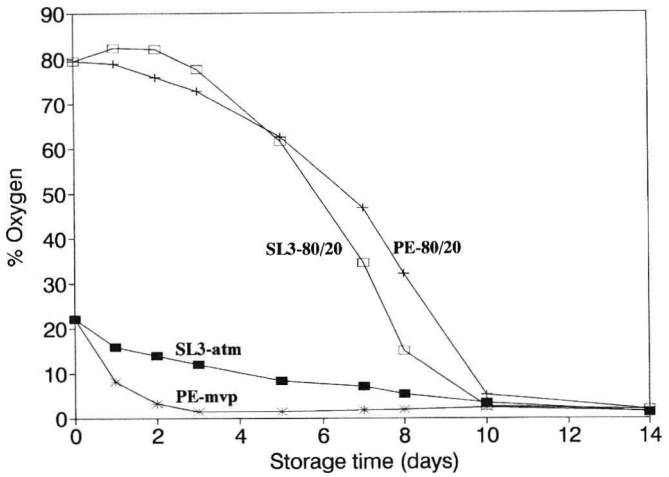

Fig. 1—Oxygen content in four MA packagings of shredded iceberg lettuce ('Roxette') during storage at 5°C. $LSD_{0.95}$ value was 0.66% O_2.

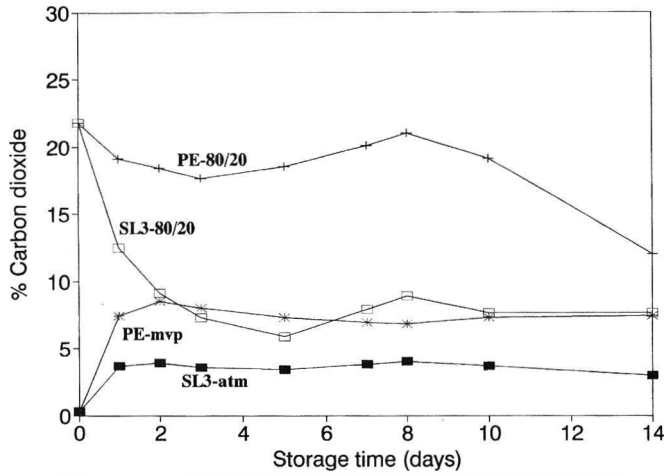

Fig. 2—Carbon dioxide content in four MA packagings of shredded iceberg lettuce ('Roxette') during storage at 5°C. $LSD_{0.95}$ value was 0.34% CO_2.

Chroma: $(a^2 + b^2)^{1/2}$

Total color difference (dE): $[(L-L_0)^2 + (a-a_0)^2 + (b-b_0)^2]^{1/2}$ where L_0, a_0 and b_0 are values from day zero.

A high degree of browning results in low hue angles and chroma values, but high dE values (Gnanasekharan et al., 1992). Before measuring, the colorimeter was calibrated with white and black standard tiles. Each sample was analyzed 10 times on each side of three bags. Degree of browning was expressed as an average of a triplicate of 20 hue angles, chroma and dE values.

Enzyme and protein assays

Extraction and polyphenoloxidase assays were carried out as described by Heimdal et al. (1994). PPO activity was expressed as nanomoles oxygen consumed/sec (nkat) under the assumption that water contained 7.4 mg of O_2/L at 30°C with a salinity (due to the buffer) of 5.8 g/L (Montgomery et al., 1964).

Ascorbic acid measurement

Iceberg lettuce (200g) was homogenized with 100 mL 0.5% oxalic acid. The homogenate was deaerated with nitrogen for 5 min. Total ascorbic acid (TAA) was measured as follows: The homogenate was filtered through filter paper (S & S 589/2) and then treated with homocysteine to reduce dehydroascorbic acid (DHAA) to AA, according to Lento et al. (1963). Excess of homocysteine was eliminated with N-ethylmaleimide and an aliquot was potentiometrically titrated with 2,6-dichlorophenolindophenol (Pongracz, 1971). The same procedure as for TAA, but without the homocysteine treatment, was used for measuring AA. DHAA was calculated as the difference between TAA and AA.

Carbohydrates

Freeze-dried material (0.5g) was ground and extracted in 25 mL water for 24 hr at room temperature (ca 23°C). The extract was filtered through paper (S & S 589/2) and then analyzed using a high pressure liquid chromatograph (HPLC) (Shimadzu) equipped with a fast carbohydrate column (300 × 7.8 mm, BIORAD), and a refractive index detector. Mobile phase was water, oven temperature 85°C, flow rate 1.0 mL/min.

Sensory analysis

An informal panel of three persons, familiar with sensory properties of shredded lettuce, evaluated for browning, flavor, off-flavor and crispness during storage using a scale of 1 (no browning, flavor, off-flavor or crispness) to 9 (extreme browning, flavor, off-flavor or crispness).

Statistical analysis

SAS software and procedures ANOVA and GLM were used for data analysis. A 2- or 3-way analysis of variance was conducted with cultivar, storage time and MA-packaging as factors.

RESULTS & DISCUSSION

Modified atmosphere

All four cultivars showed the same response to the four MA treatments in relation to content of O_2 and CO_2 during storage. Therefore, only one cultivar, 'Roxette', is shown as an example (Fig. 1 and 2).

Since O_2 is consumed in the respiration process, the concentration decreased in all treatments. PE-mvp bags reached O_2 concentrations below 5% in 2 days. The other MA treatments did not reach O_2 equilibrium during a 10-day storage. The difference in O_2 decline can be explained by the slight evacuation of the PE-mvp bags and by the difference in O_2 permeability through the films. In SL3-80/20 the O_2 concentration apparently increased at first (Fig. 1), caused by a faster diffusion rate of CO_2 out of the bags compared to O_2 (Andersen, 1994). In PE-80/20 this was not obvious because the permeability was lower. The fastest decreasing rate in 10 days was seen in treatments starting with 80% O_2, caused by high diffusion from the bags in combination with consumption in the respiration process. After 10 days these bags reached an O_2 concentration equal to the other treatments for all cultivars.

Highest permeability in SL3 bags was very distinct in the CO_2 course (Fig. 2). Equilibrium of CO_2 concentration was reached after a few days storage. For SL3-atm bags the equilibrium value on day 10 was 3–5% CO_2, depending on cultivar. For SL3-80/20 and PE-mvp the equilibrium value was 5–8% CO_2, and for PE-80/20 it was 13–19% CO_2.

Equilibrium values for O_2 and CO_2 in PE-mvp bags were the same as found for 35 µm PE bags initially flushed with 5% O_2 and 5–10% CO_2 (Ballantyne et al., 1988).

Color

Browning was expressed as hue angles because this parameter showed a better correlation with visual estimation than did chroma and total color difference (dE) (Table 1). When measuring color on inhomogeneous material like shredded lettuce shadows will influence L-values. Lightness was therefore an ineffective color characteristic for shredded lettuce, resulting in a very low correlation between score and L-values ($r^2 = 0.39$). No significant difference in development of browning for the 4 cultivars was detected by tristimulus measurements. 'Santis' is shown as an example (Fig. 3). Packaging in PE-film gave the lowest degree of browning, resulting in the lowest decrease of hue angle (Table 1). Lettuce packaged in air (SL3-atm bags) was browner than lettuce in the other MA packagings. Lettuce

Table 1—Some biochemical, physical and sensory changes in minimally processed iceberg lettuce (average of the cultivars 'Santis', 'Telda', 'Saladin', and 'Roxette') stored under four different MA conditions

Measurements	Storage (days)	MA conditions SL3-atm	SL3-80/20	PE-80/20	PE-mvp	Correlation coefficient[g] (r^2)
Visual quality[h]	0	1.0	1.0	1.0	1.0	
	3[i]	2.9	1.4	1.4	2.0	1.00
	10[j]	8.3	4.4	1.9	2.6	1.00
Color (hue angle)	0	117	117	117	117	
	3[i]	113[b]	116[a]	117[a]	115[a]	0.94
	10[j]	101[c]	109[b]	115[a]	114[a]	0.996
Color (chroma)	0	22.4	22.4	22.4	22.4	
	3[i]	19.5[b]	21.7[a]	21.8[a]	22.1[a]	0.73
	10[j]	16.7[b]	21.9[a]	21.0[a]	21.1[a]	0.73
Color (dE)	0	0.0	0.0	0.0	0.0	
	3[i]	3.8[a]	1.6[a]	1.7[a]	1.1[a]	0.65
	10[j]	7.7[a]	4.1[b]	1.9[bc]	1.5[c]	0.97
CO_2 (%)	0	0.0	21.4	21.4	0.0	
	3[i]	4.3[dk]	9.0[ck]	19.6[ak]	8.0[bk]	0.54
	10[j]	4.6[ck]	6.3[bk]	16.4[ak]	6.8[bk]	0.48
O_2 (%)	0	21.3	77.5	77.5	21.3	
	3[i]	10.7[c]	74.6[a]	68.9[b]	2.1[dk]	0.64
	10[j]	2.8[b]	2.8[b]	6.3[a]	1.9[bk]	0.17
PPO[e] (nkat/g FW)	0	217	217	217	217	
	3[i]	190[ab]	170[ab]	148[b]	192[a]	0.56
	10[j]	181[a]	145[b]	104[c]	167[b]	0.50
TAA[e] (mg/100g FW)	0	5.66	5.66	5.66	5.66	
	3	2.63[b]	3.36[a]	3.41[a]	2.50[b]	0.63
DHAA[e] (mg/100g FW)	0	0.97	0.97	0.97	0.97	
	3	0.63[b]	1.26[a]	1.45[a]	1.30[a]	0.85
TC[e] (g/100g FW)	0	1.77	1.77	1.77	1.77	
	3	1.88[a]	1.68[c]	1.81[ab]	1.78[b]	0.58
	10	1.55[a]	1.29[b]	1.46[a]	1.56[a]	0.03
Off-flavor	10	nd[f]	nd	d[f]	d	

[a-d] Means with the same letters within a row are not significantly different ($p<0.05$).
[e] PPO: polyphenol oxidase; TAA: total ascorbic acid; DHAA: dehydroascorbic acid; TC: total carbohydrates.
[f] nd: not detectable; d: detectable.
[g] Correlation between data in the row and visual quality on day 3 or 10;
[h] Estimated using a scale of 1 (nonbrowning) to 9 (extreme browning);
[i] day 2 for Santis.
[j] day 9 for Santis.
[k] equilibrium (apparently).

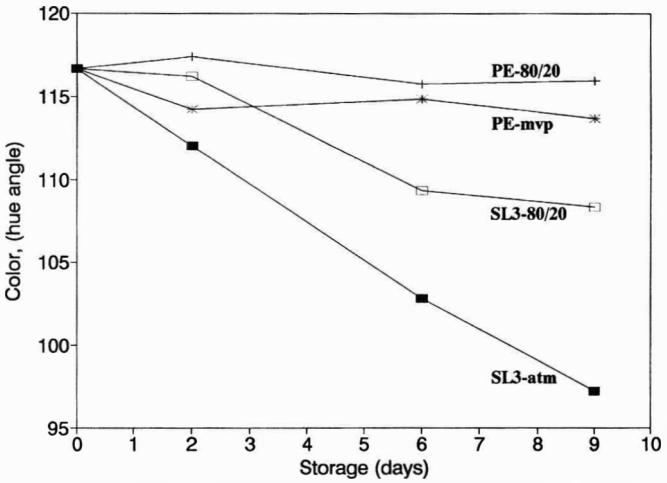

Fig. 3—Color of shredded iceberg lettuce ('Santis') during storage at 5°C, packaged in four MA conditions. A high degree of browning results in low values for hue angle. $LSD_{0.95}$ value for color (hue angle) was 2.0.

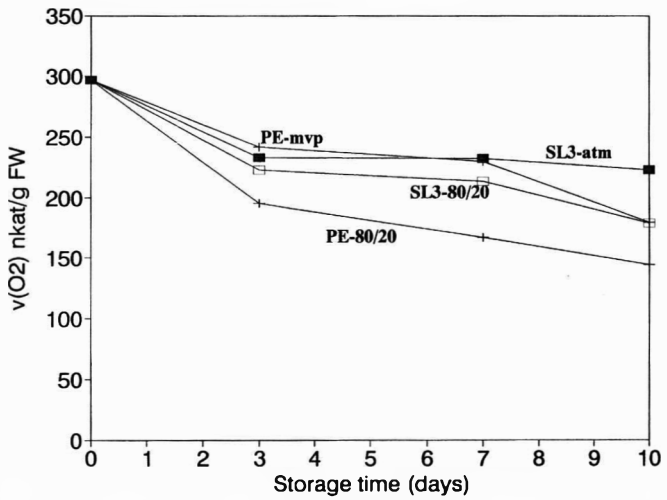

Fig. 4—Polyphenoloxidase (PPO) activity of shredded iceberg lettuce ('Saladin') during storage at 5°C, packaged in four MA conditions. $LSD_{0.95}$ value for PPO was 21.5 nkat/g FW.

packaged in SL3-80/20 bags showed more browning than that in PE-80/20 and PE-mvp bags and less browning than that in SL3-atm bags. Prevention of browning in moderate vacuum packaging has also been indicated by Gorris and Peppelenbos (1992). The highest CO_2 (Fig. 2) and the lowest browning (Fig. 3) was obtained in PE bags, in accordance with results from MA experiments with chopped lettuce by McDonald and Risse (1990). Siriphanich and Kader (1985) reported that CA storage in air + 15% CO_2 inhibited phenolic production and PPO activity in midrib segments from lettuce. However, too high CO_2 levels (>15% CO_2) must be avoided because they can cause severe CO_2 injury (Siriphanich and Kader, 1986; McDonald and Risse, 1990).

In addition, storing lettuce in low O_2 atmosphere should reduce the content of soluble phenolics and slightly inhibit PPO activity (Ke and Saltveit, 1989a). Our results demonstrated the lowest browning degree and best visual quality (PE-mvp) when an equilibrated atmosphere at 1–3% O_2 and 5–6% CO_2 was reached in the bags, in accordance with Ballantyne et al. (1988).

Polyphenoloxidase activities

PPO activity in all cultivars except 'Telda' decreased slightly during 1 wk storage. 'Saladin' is shown (Fig. 4) as an example. The cultivars 'Santis' and 'Telda' showed a lower average PPO level during storage (146 and 154 nkat/g FW) than 'Saladin' and 'Roxette' (218 and 181 nkat/g FW) ($p<0.05$), but no difference was seen in PPO response to packaging method for the four cultivars. Average PPO activity in all four cultivars during storage packaged in PE-80/20 was lower ($p<0.05$) (153 nkat/g FW) than lettuce in the other MA packagings (SL3-atm: 195 nkat/g FW; SL3-80/20: 171 nkat/g FW; PE-mvp: 193 nkat/g FW).

High CO_2 atmosphere inhibits PPO activity (Siriphanich and Kader, 1985), which was confirmed by our results. We found highest CO_2 accumulation and lowest PPO activity in PE-80/20 bags, and low CO_2 concentration in combination with higher PPO activity in SL3-atm bags (Table 1). No correlation between browning and PPO activity was found (Table 1). Hyodo et al. (1978) found no correlation between PPO activity and russet spotting (commercially important disorder, observed as numerous small brown spots along the midrib) in lettuce leaves.

Ascorbic acid

The content of TAA decreased during storage for all cultivars (Table 1). The content of TAA in fresh iceberg lettuce was lower ($p<0.001$) in 'Roxette' (4.5 mg/100g FW) than in the other cultivars (5.9–6.1 mg/100g FW).

AA degraded very quickly when lettuce was processed. One week after processing, AA was no longer detectable. Albrecht (1993) found that the retention of AA in intact heads of 8 lettuce cultivars ranged from 40–74% after 1 wk of refrigerated storage. Degradation of TAA in our results was highest in air (SL3-atm

and PE-mvp, Table 1). The initial gas mixture 80/20 delayed degradation of AA (Table 1). The high CO_2 or the high O_2 probably caused this result. In broccoli treated with short-term high CO_2 (20, 30, or 40%) loss of AA was delayed (Wang, 1979), while short-term exposure of Chinese cabbage to 10 or 20% CO_2 had no effect on AA level (Wang, 1983).

Content of DHAA increased significantly during storage (except for lettuce in SL3-atm bags) in accordance with results with intact head lettuce during storage (Böttcher, 1988). The correlation between browning and level of DHAA was high (Table 1).

Carbohydrates

No significant difference was found for the four cultivars and the average content of total carbohydrates (TC) before packaging was 1.77 g/100g FW., glucose: 48%, fructose: 52%. Under certain conditions sucrose has been detected in lettuce, as reported by Bolin and Huxsoll (1991). We found only small amounts of sucrose in a few samples, which might be caused by harvesting in the early morning (Forney and Austin, 1988). The content of TC declined slightly during storage (Table 1), indicating a consumption via respiration. After 10 days storage the decrease was 10–17% for glucose ($p<0.001$) and 6–17% for fructose ($p<0.001$) in 'Saladin' and 'Roxette'. Packaging in SL3-80/20 resulted in a lower content of TC after 10 days storage than packaging in the three other MA-conditions (Table 1). This might be caused by higher respiration. Kubo et al. (1990) found increased respiration when exposing lettuce to extreme high CO_2 atmosphere (60%). The highest CO_2 accumulation we found was 20% in PE-80/20 bags (Fig. 2). That probably was too low to influence respiration. No correlation was found between browning and TC (Table 1).

Sensory quality

Lettuce decreased in quality after processing, as expected. The best visual quality during 10 days storage was in PE-mvp bags. Lettuce in PE-80/20 bags had an overall poor sensory quality after 10 days storage because of development of off-flavor and tissue softening. Poor visual quality soon occurred in SL3-atm bags because of browning, but no off-flavor developed during storage. The loss of lettuce flavor was highest when stored in PE for 'Telda' and 'Saladin'. After 10 days an off-flavor was recognized in PE bags. After 17 days following processing, a fruity flavor developed in PE-mvp bags, and a butanoic acid flavor was recognized in 80/20 bags. Mateos et al. (1993) found that high CO_2 resulted in development of fermentative products such as ethanol and acetaldehyde in intact lettuce heads. In addition, very low O_2 concentration in the bags increased anaerobic respiration and resulted in low sensory quality because of off-flavors (McDonald and Risse, 1990).

Instead of browning, a yellowing of leaves occurred in the 80/20 bags, which might be a result of chlorophyll loss. This was in accordance with Satler and Thimann (1983), who found that loss of chlorophyll in oat leaves was greatly increased by 100% O_2.

Relationships between browning, MA-conditions and lettuce constituents

Shredded lettuce in PE-mvp bags had a high visual quality after 10 days storage. The optimal equilibrium atmosphere (2% O_2 and 7% CO_2) was obtained in these bags after a few days storage. Low O_2 concentrations delay enzymatic browning because PPO has a relatively low affinity for oxygen (Burton, 1974). In addition, AA may have acted as a better antioxidant in PE-mvp bags since DHAA reached a higher value (Table 1) than in the SL3-atm bags. Because of the low O_2 content in PE-mvp bags, some off-flavor was detected.

Severe browning occurred in lettuce in SL3-atm bags after a few days storage (Table 1). In agreement with the theory of enzymatic browning, we found the highest PPO activity in lettuce from these bags (Fig. 4 and Table 1). In addition, AA may not have acted as an efficient antioxidant because of the fast degradation of TAA and the low DHAA content after 3 days storage (Table 1).

The high O_2 level in bags initially flushed with 80% O_2 and 20% CO_2 stimulated respiration as measured by accelerated loss of carbohydrates (Table 1). No browning occurred despite the high O_2 concentration. The high CO_2 and/or O_2 level may have inhibited some enzyme systems or accelerated processes that converted the substrates into colorless products (Ke and Saltveit, 1989b). Despite the absence of enzymatic browning in 80/20 treatments, the quality of the lettuce in those bags was poor after 10 days of storage. This occurred in PE-80/20 bags because of off-flavor and tissue softening.

CONCLUSION

ENZYMATIC BROWNING of shredded iceberg lettuce was inhibited for at least 10 days by moderate vacuum packaging in 80 µm polyethylene bags. Storage time exceeding ten days should be avoided due to increasing off-flavor in bags with good visual quality. Enzymatic browning of shredded lettuce can be followed by colorimetric measurements, whereas polyphenol oxidase activity showed no simple relationship to browning.

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Aroma Changes in Fresh Bell Peppers (*Capsicum annuum*) after Hot-air Drying

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ABSTRACT

The aroma of fresh and hot-air dried bell peppers (*Capsicum annuum*) was evaluated by sensory and instrumental methods. Hot-air drying decreased levels of the odor compounds (*Z*)-3-hexenal, 2-heptanone, (*Z*)-2-hexenal, (*E*)-2-hexenal, hexanol, (*Z*)-3-hexanol, (*E*)-2-hexenol, and linalool, which have green, vegetable-like, fruity, and floral notes, while intensity scores of corresponding sensory aroma attributes also decreased. The aroma of rehydrated dried samples was mainly characterized as savory, rancid/sweaty, sweet/sickly, hay-like, cacao, caramel, and nutty. Drying increased the levels of 2-methylpropanal, 2- and 3-methylbutanal, which have cacao, spicy, and rancid/sweaty odor notes; these volatiles may be correlated with the corresponding aroma attributes in the dried fruits. Principal component analysis demonstrated relationships between instrumental and sensory data for fresh samples, whereas they were more complex for dried fruits.

Key Words: *Capsicum annuum*, bell pepper, gas chromatography, aroma, sensory evaluation

INTRODUCTION

CAPSICUM ANNUUM belongs to the Solanaceae family. The fruits are used fresh or processed for color, pungent character (i.e., capsaicinoids), and typical aroma and taste (Somos, 1984; Govindarajan, 1985). Aroma and taste have been considered less important for fresh and processed *Capsicum* fruits than color and pungency (Govindarajan et al., 1987). Breeding programs are mainly focused on disease resistance, crop yield, carotenoid levels, and nutritional value (vitamin C content) (Somos, 1984; Minguez-Mosquera et al., 1994). However, the growing "fresh" market and the increasing use of dried vegetables in convenience foods has made aroma a quality parameter (Feinberg, 1973; Govindarajan et al., 1987; Zachariasse and Abrahamse, 1993).

Several studies have included the flavor evaluation of different fresh and processed *Capsicum* varieties. Chitwood et al. (1983) observed that "green" aroma attributes discriminated different cultivars of fresh California *Capsicums*. Luning et al. (1994b) studied the flavor evaluation of fresh bell peppers at different ripening stages, perceived while eating. Sweetness, sourness, and red bell pepper aroma were characteristic attributes for ripe red stages, and bitterness and "green" flavor notes, such as grassy, herbal, cucumber, and green bell pepper aroma, characterized immature green stages. The aroma profiles of commercial dried Hungarian Edelsüss and Spanish *Capsicums* were studied by Wilkins (1994). The Spanish *Capsicum* scored relatively high for off-aroma, rubbery, and sharp, while the Hungarian samples generally scored higher for tomato, hay, and sweet aroma. Van Ruth and Roozen (1994) showed that rehydrated dried Chilean, Turkish, and Hungarian bell peppers were more different in taste attributes like sour, bitter, and pungency than in aroma attributes. Drying of fresh bell peppers greatly changed the composition of volatile compounds; most volatiles evaporated and new volatile odor compounds were formed by chemical reactions (Luning et al., 1994a; Van Ruth and Roozen, 1994). Such changes in volatiles might affect the aroma of fresh peppers after drying.

Information on changes in aroma of fresh bell peppers by hot-air drying as related to changes in composition of volatile odor compounds is limited. Our objective was to characterize the aroma of both fresh and hot-air dried bell peppers of four different colors (i.e., green, red, white, and yellow), and to evaluate differences due to drying.

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MATERIALS & METHODS

Materials

Bell peppers of three commercial Dutch cultivars i.e., cv. Mazurka, cv. Blondy, and cv. Kelvin were grown under greenhouse conditions as reported by Luning et al. (1994a). Immature green fruits of cv. Mazurka were harvested 6 wks after fruit set. The ripe red fruits of cv. Mazurka, the ripe white fruits of cv. Blondy and ripe yellow fruits of cv. Kelvin were harvested at about 10 wk after fruit set. Fruits (40) of each color were collected in the early morning and stored at 13°C for a maximum of 5 days. Bell peppers (20) of each color were used for isolation of volatile compounds, and the remaining ones were used for sensory evaluation of fresh vegetables.

Dried bell pepper samples were prepared by drying 40 peppers of each color in a pilot-scale hot-air tray dryer (Bronswerk Heat Transfer B.V., Nijkerk, The Netherlands). Bell peppers were washed, deseeded, and cut into pieces of 1 cm^2. Subsequently, the samples were dried at 65°C until a final moisture content of 0.1 kg H_2O/kg dry matter was obtained. Dried samples were stored in the dark, under vacuum at 4°C until analysis of volatiles and sensory evaluation (up to 2 mo).

All chemicals were obtained from Jansen Chimica (Tilburg, The Netherlands).

Sensory analysis

Sensory descriptive analysis was used to evaluate aroma attributes of fresh and hot-air dried peppers as described by Stone and Sidel (1993).

Sample preparation and presentation. Fresh bell peppers of each color were cleaned, then the top and bottom parts and seed lobes were removed. The parts were ground in a household mixer (Quick Foodmaster, Tefal) and 30-g portions of crushed samples were immediately transferred into glass jars and closed with odorless caps. Hot-air dried bell peppers were rehydrated with water at 80°C for 10 min and cooled to room temperature (≈23°C). Preliminary experiments revealed that up to 60% of the total evaporated water was absorbed. Portions of dried bell pepper (3g each) were crushed in a mortar and subsequently rehydrated at 80°C in the glass jars.

Training of panel. Fifteen assessors, aged 25 to 35 years, and with extensive sensory evaluation experience on other fruits and vegetables, were selected for aroma evaluation of fresh and hot-air dried bell peppers. Panel training was executed in different phases. In the first phase, 20 chemicals dissolved in water were sniffed and described (Table 1). Successively, the same chemicals were evaluated using a checklist including the compiled descriptors, to train odor recognition of the assessors. In the next sessions, an assortment of fresh bell pepper cultivars with different aromas were provided as a homogenate to the panel for a wide range of perceived sensory properties, to be expected in the fresh samples. These cultivars were also provided as freeze-dried and hot-air dried samples to generate sensory attributes for the processed peppers. The same assortment of fresh and processed cultivars was used for training the intensity scaling of perceived attributes over a period of 1 mo. Finally, the assessors made suggestions and selected attributes for the panel, to describe aroma characteristics of fresh and dried bell peppers; two lists of consensus attributes were derived (cf. Tables 3 and 4).

Table 1—Descriptors and reference chemicals used for panel training

Reference chemical	Amount in 10 mL water	Major descriptor
Linalool	1.5 mL	Floral, fragrant
Dimethylsulfide	30.0 µL	Sulfury, gassy
α-Limonene	2.4 mL	Fruity, citric
α-Pinene	0.7 mL	Woody/pine-like
Myrcene	5.0 µL	Geranium leaves
(*E*)-2-hexenal	10.0 µL	Almond-like
(*Z*)-3-hexenol	10.0 µL	Grassy, herbal
Onion oil	10.0 µL	Onion, garlic
Formic acid	0.3 mL	Pungent, sharp
Soap/water solution	0.1 mL	Soapy
Trimethylamine	30.0 µL	Fishy
Butyl acetate	10.0 µL	Fruity (ester-like)
Ethyl octanoate	10.0 µL	Fruity (other)
1-Octene-3-ol	10.0 µL	Mushroom
Vanillin	0.1 µg	Sweet, caramel
Phenol	10.0 µL	Rubbery
Hexanal	1.0 µL	Grassy, green vegetable
2-Isobutyl-3-methoxy-pyrazine	1.0 µL	Green bell pepper
Butyric acid	0.6 mL	Rancid, sweaty
Methyl salicylate	10.0 µL	Winter green-, menthol-like
		Aroma of fresh and dried *Capsicum* fruits
2-Methylbutanal	20.0 µL	Cacao, sweaty

Sensory evaluation. For assessment of aroma, all samples were presented in coded jars covered with aluminum foil, at room temperature in isolated air-conditioned booths with slight positive pressure. Fresh and hot-air dried samples of each colour (green, red, yellow, and white) were evaluated three times. Aromas of four fresh or four dried peppers were evaluated at each session. Samples were assesed in the morning and afternoon, using a balanced complete block design. Judges indicated the intensity of each attribute by an unstructured line scale anchored with 0 and 100 at the ends. Data were acquired by the computerized PSA-SYSTEM (OP&P, Utrecht, The Netherlands).

Gas chromatography

Details of the isolation procedure of volatile bell pepper compounds, analysis and quantification by gas chromatography (GC), and identification by GC-mass spectrometry (GC-MS) were described previously (Luning et al., 1994a). Five fresh fruits of each color were sliced into small pieces and 45-g portions were homogenized with 50 mL water in a Waring blendor. Weights of dried samples were taken relative to dry matter contents of the 45-g portions of fresh peppers. Dried samples were rehydrated with 80°C water for 15 min in closed flasks and cooled on ice to room temperature prior to homogenization with 50 mL water in a Waring blendor. Volatile compounds were isolated from homogenized fresh and rehydrated samples, by flushing with 30 mL/min purified nitrogen for 2 hr at room temperature (three replicates). Detection and description of odor active compounds in the GC-effluent were carried out by an experienced sniffing port panel as reported previously (Linssen et al., 1993; Luning et al., 1994a). Peak areas of identified odor active compounds were expressed as percentage of total extracted peak area (Volt × sec).

Data analysis

GC peak areas (percentage of total peak area) of the odor compounds and mean scores of each sensory attribute were subjected to analysis of variance (ANOVA, Genstat-5), to determine least significant differences (LSD) among colors (one-way ANOVA) and between fresh and rehydrated hot-air dried samples (two-way ANOVA) (Table 2). Principal component analysis (PCA) was applied to the combined data set of percentage peak areas and mean score ratings. This enabled study of relationships between instrumental and sensory data of respectively fresh and hot-air dried bell peppers (Piggot and Sharman, 1986). PCA was carried out with UNSCRAMBLER analytical software (OP&P, Utrecht, The Netherlands). In addition, combined data sets were subjected to partial least-squares regression (PLS) as described (Martens and Martens, 1986).

RESULTS & DISCUSSION

Sensory evaluation of fresh and hot-air dried peppers

Results were compared for analysis of variance of descriptive analysis data for each attribute for fresh and dried samples. Highly significant differences occurred among fresh samples for all attributes, while less attributes were different among dried samples. Although inconsistent assessors were eliminated during selection, assessors were an important source of variation in all attributes due to the nature of sensory evaluation and individual differences. Only a few significant assessor-by-replicate and sample-by-replicate interactions occurred.

Drying of green and red cv. Mazurka, white cv. Blondy, and yellow cv. Kelvin peppers produced completely different aromas as compared to fresh fruits. Generation of descriptors for aroma evaluation resulted in different lists for fresh and dried samples (Tables 3 and 4). The aroma perception of both fresh unripe green and ripe white bell peppers was dominated by fresh green aroma attributes; intensity scores for herbal, grassy, cucumber, and fruity/fresh were higher ($P < 0.05$) than for ripe red and yellow peppers. In addition, white fruits had highest scores for floral and cucumber, and green fruits for herbal, grassy, and green bell pepper. The aroma of ripe red and yellow fruits was

Table 2—Analysis of variance of attribute ratings in fresh and dried samples

	F ratios													
	Sample(S)		Assessor(A)		Replicate(R)		S×A		A×R		S×R		MSE[b]	
Attribute	F[a]	D	F	D	F	D	F	D	F	D	F	D	F	D
Herbal	20.8***	1.1	16.6***	17.9***	1.6	1.9	3.8***	0.9	0.7	1.9	1.0	1.0	55.8	77.0
Green bell pepper	79.6***	1.1	11.7***	23.2***	1.7	0.5	2.3***	1.0	1.2	1.1	1.9	0.8	117.1	43.3
Grassy	52.7***	1.1	9.3***	19.7***	6.4**	4.1	1.5*	1.5	0.7	1.2	3.1**	0.7	199.8	35.9
Cucumber	17.9***	5.9***	16.6***	39.9***	3.7*	10.9*	2.6***	1.5*	2.0**	4.3**	1.0	0.7	94.6	30.8
Floral	12.9***	4.1**	10.6***	27.9***	0.6	2.9	1.9**	1.6*	1.6	1.2	1.8	0.6	124.5	34.4
Fruity/fresh	24.4***	4.2*	12.0***	9.1***	0.1	0.6	2.9***	2.2*	1.3	1.4	4.5**	0.9	204.4	84.0
Resinous	4.6**	1.1	32.0***	13.9***	0.3	2.1	1.9**	1.1	1.4	1.6	1.2	1.2	28.6	26.2
Rubbery	33.3***	9.9***	34.1***	6.9***	2.7	1.0	3.2***	2.7***	3.1**	0.8	6.2**	0.2	108.6	52.5
Rancid/sweaty	44.4***	1.1	23.2***	14.3***	8.4**	1.6	3.7***	0.9	1.3	1.4	6.1**	0.5	45.1	134.1
Sweet/sickly	8.6***	0.6	9.7***	23.6***	1.1	0.3	1.8**	0.7	1.1	0.9	1.8	1.9	110.4	170.0
Fruity/chemical	10.9***	3.3*	29.2***	6.9***	1.0	30.0**	3.9***	1.5*	1.2	1.3	0.9	2.4*	98.9	72.3
Musty/earthy	9.7***	0.6	22.6***	11.9***	0.5	6.0	2.5***	1.4	0.9	2.0	3.1**	0.6	23.6	77.8
Sharp	9.5***	1.8*	44.5***	14.8***	2.9	0.6	4.0***	1.8**	2.6**	1.7*	1.1	1.7	81.5	111.1
Spicy	3.0**	0.7	17.1***	31.3***	1.7	2.0	2.2**	1.6	1.1	1.1	1.9	1.1	91.6	58.2
Hay-like	5.0**	2.2**	37.1***	19.4***	1.2	1.3	4.6***	2.0**	1.5	1.1	0.5	0.5	13.8	152.4
Cacao	n.e.[c]	17.1***	n.e.	6.0***	n.e.	0.5	n.e.	2.8***	n.e.	1.3	n.e.	0.4	n.e.	51.3
Caramel	n.e.	12.7***	n.e.	19.5***	n.e.	1.7	n.e.	3.8***	n.e.	1.1	n.e.	1.7	n.e.	45.9
Nutty	n.e.	12.6***	n.e.	21.2***	n.e.	0.7	n.e.	4.9***	n.e.	0.9	n.e.	2.6*	n.e.	38.3
Savory	n.e.	2.7*	n.e.	20.4***	n.e.	0.1	n.e.	1.7**	n.e.	0.9	n.e.	0.9	n.e.	116.9
Dried tomato	n.e.	6.9***	n.e.	13.0***	n.e.	1.3	n.e.	3.1***	n.e.	0.8	n.e.	0.8	n.e.	51.0

[a] Fresh (F) and hot-air dried (D) sample.
[b] MSE: mean squares error.
[c] n.e. not evaluated.
***,**,* Significantly different at $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively.

Table 3—Mean score ratings[a] of sensory attributes of fresh green and red cv. Mazurka (GM, RM), white cv. Blondy (WB), and yellow cv. Kelvin (YK)

Attribute	Code	GM	WB	RM	YK
Herbal	HER	33.3[a]	19.2[b]	12.0[c]	10.5[c]
Green bell pepper	GBP	32.4[a]	14.9[b]	9.8[b]	10.4[b]
Grassy	GRA	13.8[a]	9.9[b]	5.5[c]	4.7[c]
Cucumber	CUC	14.6[a]	22.5[b]	7.6[c]	9.5[c]
Floral	FLO	13.5[a]	23.5[b]	11.7[a]	9.6[a]
Fruity/fresh	FRF	30.8[a]	40.0[b]	19.2[c]	17.8[c]
Resinous	RES	5.6[a]	4.2[a]	3.8[ab]	2.2[b]
Rubbery	RUB	13.0[a]	10.2[a]	33.9[b]	27.6[b]
Rancid/sweaty	RAN	5.7[a]	4.2[a]	17.8[b]	9.6[c]
Sweet/sickly	SIC	10.4[a]	9.2[a]	18.7[b]	15.8[b]
Fruity/chemical	FRC	6.5[a]	13.4[b]	19.5[c]	14.5[b]
Musty/earthy	MUS	3.6[a]	3.3[a]	11.4[b]	6.9[a]
Sharp	SHA	18.6[a]	10.3[b]	17.7[ac]	13.8[bc]
Spicy	SPI	11.0[ab]	7.3[b]	13.3[a]	11.8[ab]
Hay-like	HAY	5.7[a]	2.9[b]	4.8[ac]	3.8[bc]

[a,b,c] Mean scores (n=3) in row with different letters are significantly different ($P < 0.05$).

Table 4—Mean score ratings of sensory attributes of hot-air dried green and red cv. Mazurka (GM, RM), white cv. Blondy (WB), and yellow cv. Kelvin (YK)

Attribute	Code	GM	WB	RM	YK
Cacao	CAC	11.6[a]	7.9[b]	3.0[c]	3.2[c]
Caramel	CAR	8.0[a]	7.2[a]	4.3[b]	3.4[b]
Nutty	NUT	9.3[a]	13.4[a]	3.7[b]	4.7[b]
Savory	SAV	15.6[a]	15.3[a]	11.9[ab]	9.3[b]
Dried tomato	TOD	7.8[a]	4.8[b]	11.1[c]	6.6[ab]
Herbal	HER	6.5	5.8	5.1	4.0
Green bell pepper	GBP	7.7	7.2	6.2	6.1
Grassy	GRA	8.7	6.4	5.5	6.2
Cucumber	CUC	6.5[a]	11.1[b]	7.5[a]	9.1[b]
Floral	FLO	4.4[a]	4.9[a]	6.2[ab]	8.6[b]
Fruity/fresh	FRF	6.1[a]	6.1[a]	9.4[b]	12.3[c]
Resinous	RES	6.7	8.5	5.1	4.4
Rubbery	RUB	4.5[a]	4.5[a]	12.3[b]	6.4[a]
Rancid/sweaty	RAN	10.3	10.6	10.2	12.4
Sweet/sickly	SIC	16.6	17.4	20.0	18.7
Fruity/chemical	FRC	6.2[a]	9.6[a]	10.0[ab]	14.1[b]
Musty/earthy	MUS	6.9	8.3	7.2	4.3
Sharp	SHA	9.0[ab]	9.6[ab]	12.2[a]	7.5[b]
Spicy	SPI	8.4	7.3	10.5	7.9
Hay-like	HAY	16.0[a]	20.1[a]	10.2[b]	8.7[b]

[a,b,c] Mean scores (n=3) in rows with different letters are significant y different ($P < 0.05$).

mainly characterized by higher scores for rubbery, rancid/sweaty, and sweet/sickly; the red bell peppers had also high scores for musty. The fruity/chemical aroma appeared to be characteristic for all ripe fruits (Table 3). Similarly, Chitwood et al. (1983) reported "green" aroma characteristics, like grassy, green and garbanzo bean to describe the aroma of different *Capsicum* cultivars. Moreover, floral, rose, and apple appeared to be also important contributors to the aroma of these *Capsicums*. However, they reported no aroma attributes like rancid/sweaty, sweet/sickly and/or rubbery.

After drying, five other attributes, i.e., cacao, caramel, nutty, savory (i.e., spicy, salty), and dried tomato, were necessary to describe the aroma of the pepper samples; these were not relevant in the fresh samples (Table 4). Obviously, all dried samples were characterized by relatively high scores for savory, rancid/sweaty, sweet/sickly, and hay-like (Table 4). Furthermore, intensity scores for cacao, caramel, and nutty were higher ($P < 0.05$) in green and white peppers. Dried tomato and rubbery aroma scored highest in red, and fruity/chemical and fruity/fresh aroma rated highest in yellow peppers. Wilkins (1994) reported similar odor attributes like rubber, tomato, and hay for evaluation of dried Hungarian and Spanish *Capsicum* cultivars.

Drying changed not only the main aroma attributes of fresh fruits, but also the perceived aroma intensity. Overall the intensity scores were lower in dried than in fresh samples (Tables 3 and 4). Mean scores of corresponding attributes for fresh and dried fruits were subjected to ANOVA. Some attributes changed

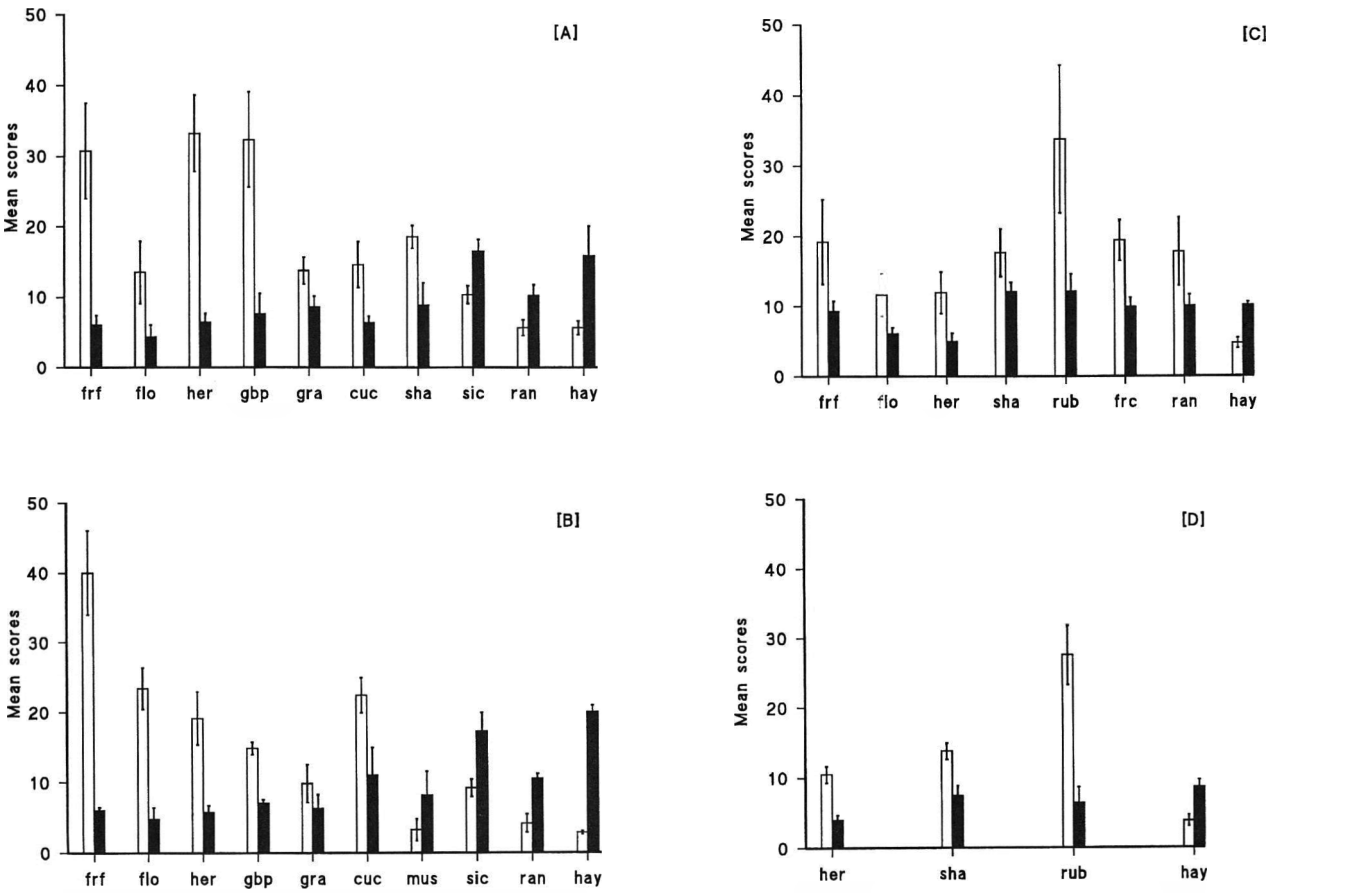

Fig. 1—Intensity scores of aroma attributes ($P < 0.05$) due to drying of fresh (open bars) green (A), white (B), red (C), and yellow (D) bell peppers to dried fruits (filled bars). (See codes, Tables 3 and 4.)

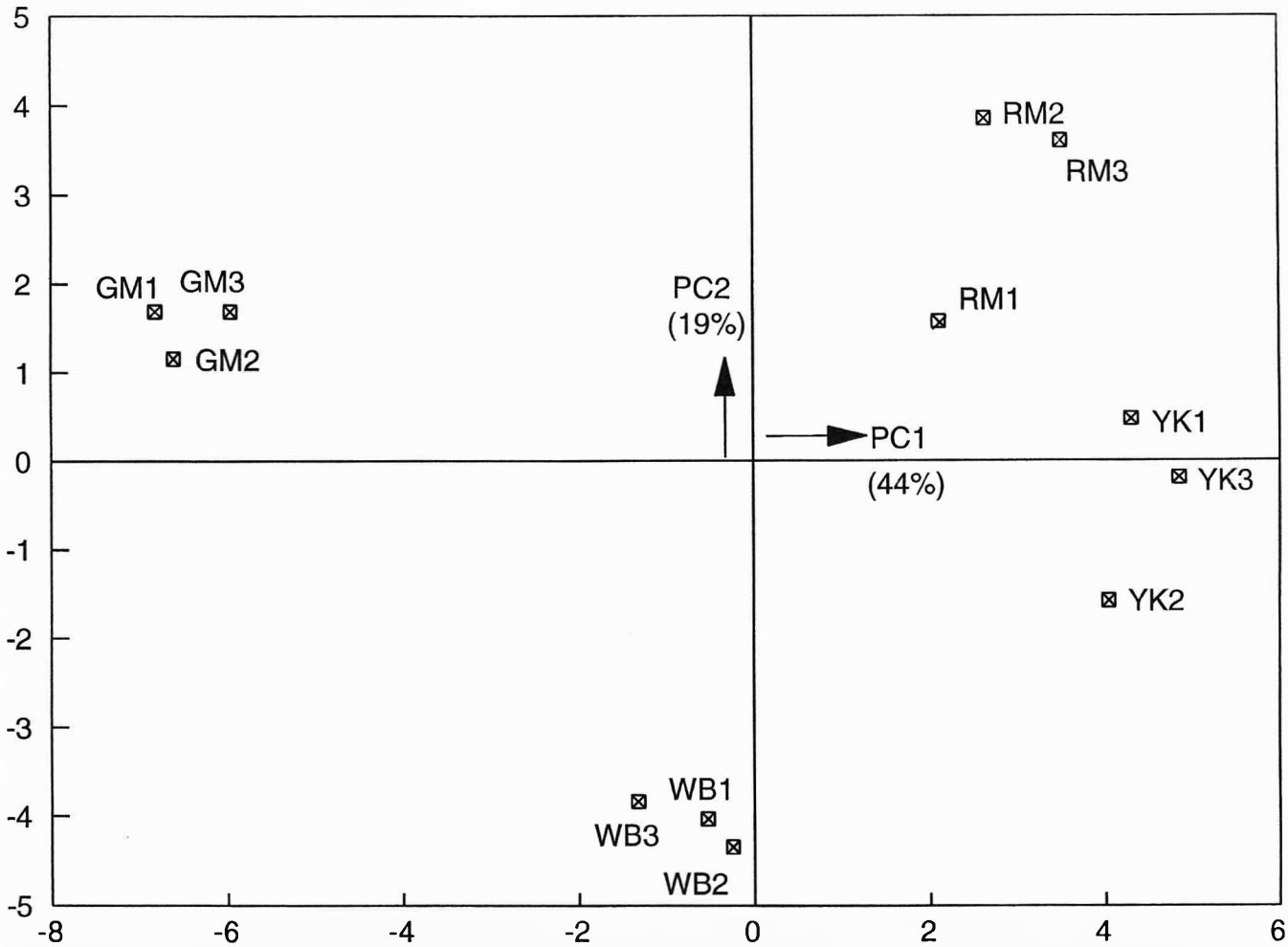

Fig. 2—PCA sample scores of fresh green and red cv. Mazurka, white cv. Blondy, and yellow cv. Kelvin. (Codes, Table 3.)

Table 5—Percentage peak areas[a] of volatile odor compounds isolated by dynamic headspace analysis from fresh and hot-air dried green and red cv. Mazurka (GM, RM), white cv. Blondy (WB), and yellow cv. Kelvin (YK)

				Fresh				Dried			
No	Compound	Odor descriptions[b]	Sign[c]	GM	WB	RM	YK	GM	WB	RM	YK
1	2-Methylpropanal	Cacao, spicy, sweaty	y,z	0.00	0.00	0.00	0.00	2.62	2.60	1.59	1.50
2	2-Methylbutanal	Cacao, spicy, sweaty, rotten/cv[d]	x,y,z	0.04	0.06	0.08	0.06	13.03	19.44	14.24	12.51
3	3-Methylbutanal	Cacao, spicy, sweaty, rotten/cv	x,y,z	0.04	0.04	0.06	0.08	16.00	23.01	29.92	25.28
4	2,3-Butanedione	Caramel, sweet	x,y,z	0.04	0.19	0.06	0.28	0.60	0.30	0.53	0.43
5	1-Penten-3-one	Chemical/pungent, spicy	x,y,z	1.35	0.63	0.81	0.76	1.45	0.58	4.94	1.83
6	2,3-Pentanedione	Fruity, sweet, caramel	z	0.00	0.00	0.00	0.00	0.09	0.06	0.10	0.08
7	Hexanal	Grassy, lettuce, green bp[e]	x,y,z	29.44	10.93	11.73	11.57	16.27	17.07	11.89	15.55
8	3-Carene	Red bp, rubbery, green bp	x,z	0.09	0.30	0.37	0.48	0.27	0.26	0.45	0.34
9	(*Z*)-3-hexenal	Lettuce, grassy, green bp, fruity	x,z	10.73	0.51	0.20	0.00	0.00	0.00	0.00	0.00
10	2-Heptanone	Fruity, Spicy, red bp	x,y,z	0.32	0.95	1.66	3.62	0.60	0.82	0.63	1.51
11	*o*-Xylene	Rubbery, spicy, geranium	x,y,z	0.16	0.69	0.75	1.26	0.58	0.59	0.99	1.06
12	(*Z*)-2-hexenal	Grassy, lettuce, green bp	x,z	0.86	0.16	0.35	0.35	0.13	0.10	0.14	0.20
13	(*E*)-2-hexenal	Almond, fruity, sweet	x,y,z	12.48	8.07	36.38	1.80	1.46	0.35	1.84	0.74
14	(*Z*)-β-Ocimene	Rancid, sweaty	x,y,z	0.14	0.27	0.33	0.22	0.52	0.22	0.28	0.06
15	Octanal	Fruity	x,z	0.15	0.57	0.87	1.16	0.49	0.39	0.50	0.58
16	4-Octen-3-one	Mushroom	x,y,z	0.02	0.08	0.12	0.26	1.57	0.81	0.95	1.19
17	6-Methyl-5-hepten-2-one	Sweaty, musty	x,y,z	0.14	0.64	0.85	0.86	0.57	0.35	0.86	0.98
18	Hexanol	Fruity, lettuce, rotten/cv	x,y,z	2.61	1.86	1.66	0.97	0.46	0.25	0.22	0.10
19	(*Z*)-3-Hexenol	Grassy, lettuce, cucumber	x,y,z	6.72	3.68	0.07	0.11	0.23	0.08	0.00	0.00
20	Nonanal	Mushroom, herbal	x,z	1.07	2.61	2.39	4.01	2.00	1.30	2.32	2.21
21	(*E*)-2-Hexenol	Almond, spicy, geranium, red bp	x,y,z	0.27	0.55	7.50	0.59	0.21	0.16	0.24	0.33
22	(*E*)-2-Octenal	Almond, sweet, herbal	x,y,z	0.10	0.20	0.26	0.35	1.08	0.84	0.85	0.85
23	2-Isobutyl-3-methoxypyrazine	Green bp, red bp, lettuce	x,y,z	0.34	0.51	0.53	0.76	0.96	0.90	0.51	0.69
24	(*E*)-2-Nonenal	Cucumber, green bp, carrot	x,y,z	0.05	1.36	0.16	0.23	1.12	1.75	0.40	0.96
25	Linalool	Floral	x,y,z	0.10	0.27	0.07	0.10	0.12	0.07	0.00	0.00
26	(*E,Z*)-2,6-Nonadienal	Cucumber, lettuce	x,y,z	0.02	0.13	0.11	0.23	0.19	0.25	0.12	0.20
	Sum percentage peak areas			67.2	35.3	67.4	30.1	62.6	72.5	74.5	69.5
	Total Peak area of extracted volatile compounds			324.8	184.2	125.8	71.4	85.1	94.2	80.8	62.1

[a] Average percentage peak areas of three experiments; mean coefficient of variation is 15%.
[b] Odor descriptions obtained previously by sniffing port analysis (Luning et al., 1994 a,c).
[c] Significance of differences at $P < 0.05$; x, significant differences within fresh samples; y, within dried samples; and z, between fresh and dried samples of the same color.
[d] cv = cooked vegetable.
[e] bp = bell pepper.

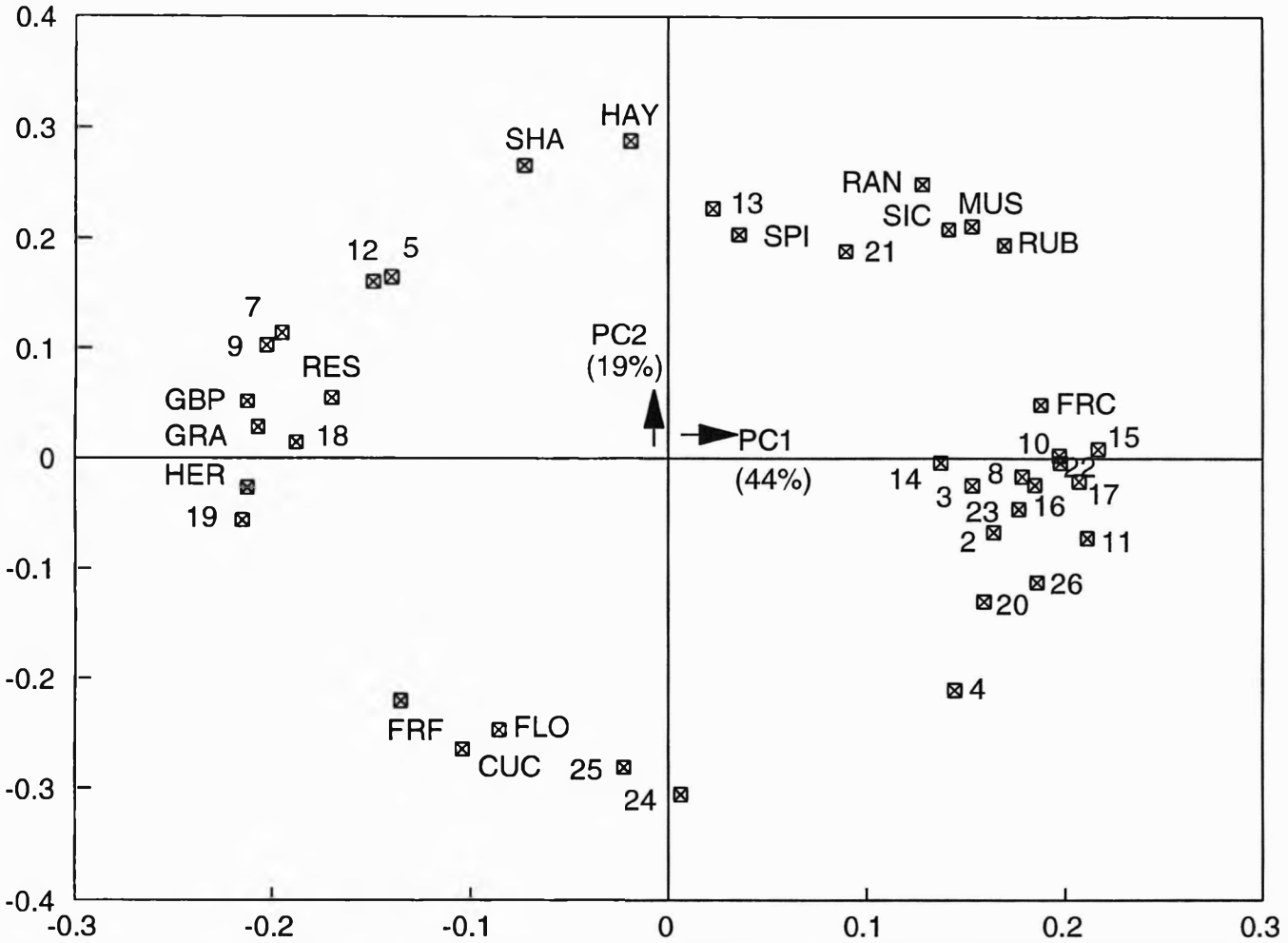

Fig. 3—PCA loadings of aroma attributes and percentage peak areas of odor compounds of fresh bell peppers. (Codes, Tables 3 and 5.)

($P < 0.05$) upon drying (Fig. 1). Obviously, in all samples scores for hay-like aroma increased while herbal aroma decreased. Intensity scores of all "green" attributes were lower (Fig. 1 A+B) in dried green and white samples, whereas scores for sweet/sickly and rancid aroma increased; musty aroma increased only in the white fruits (Fig. 1 B). Intensity of fruity/fresh and floral aroma notes decreased in green, white, and red but not in yellow samples. The dried green, red, and yellow fruits (Fig. 1 A, C, and D) scored lower on sharp aroma, but dried red and yellow fruits only scored lower on rubbery aroma.

Volatile odor active compounds of fresh and hot-air dried peppers

Volatile compounds were extracted from homogenized fresh and rehydrated dried bell peppers by a dynamic headspace isolation, trapped on Tenax, and thermally desorbed. Odor active compounds were detected and characterized previously by an experienced GC-sniffing port panel and identified by GC/MS (Luning et al., 1994a,c). The percentage peak areas of 26 identified odor active compounds were compared (Table 5) along with the total peak area of extracted volatile compounds, and the sum of percentage peak areas of odor active volatiles. The proportional contribution of odor active compounds to the total extracted volatiles was higher in the fresh green and red than fresh yellow and white bell peppers, whereas it was about the same in all dried samples (Table 5). Hot-air drying decreased the total amount of extracted volatile compounds, and also changed ($P < 0.05$) proportions between volatile compounds (Table 5). The percentage peak areas of (*Z*)-3-hexenal (9), 2-heptanone (10), (*Z*)-2-hexenal (12), (*E*)-2-hexenal (13), hexanol (18), (*Z*)-3-hexanol (19), and (*E*)-2-hexenol (21), and linalool (25), which had green, vegetable-like, fruity, and floral characteristics, decreased ($P < 0.05$) during drying in most of peppers. Probably, these changes were responsible for decreased intensity scores of corresponding green, fruity, and floral sensory aroma attributes in dried samples (Fig. 1 A–D). In all dried samples the major odor active compounds were 2-methyl-propanal (1), 2- and 3-methylbutanal (2,3), hexanal (7), and nonanal (20). The first three volatiles had typical cacao, spicy, sweaty, and rotten/cooked vegetable odor notes, while nonanal had more spicy and herbal characteristics (Table 5). Moreover, the percentage peak areas of 2,3-butanedione (4, caramel, sweet), 2,3-pentanedione (6, fruity, sweet, caramel), 4-octen-3-one (16, mushroom), (*E*)-2-octenal (22, almond, sweet), and (*E*)-2-nonenal (24, cucumber, green bell pepper) were higher ($P < 0.05$) in the dried peppers. Several compounds had cacao, spicy, sweaty/rancid, and sweet odor characteristics and may contribute to sensory aroma attributes like savory, sweet/sickly, rancid/sweaty, cacao, and caramel, characteristic for all dried samples (Table 4) as compared to fresh fruits (Table 3). In addition, although hay-like was a typical sensory aroma attribute for all dried samples, no odor compounds with hay-like notes were observed by the GC-sniffing port panel. Leino (1992) reported that the large relative proportions of carbonyl compounds like 2-methyl-2-pentenal, hexanal, 2-methyl-2-butenal, nonanal, and 2-hexenal, and high intensity scores for hay-like aroma were characteristic for hot air-dried chives (40°C) as compared to fresh products. Moreover, Wilkins (1994) demonstrated that hexanal correlated highly with hay, tomato and off-aroma of dried Hungarian *Capsicums*, but suggested that these aroma notes may be the result of extensive interactions between odor compounds.

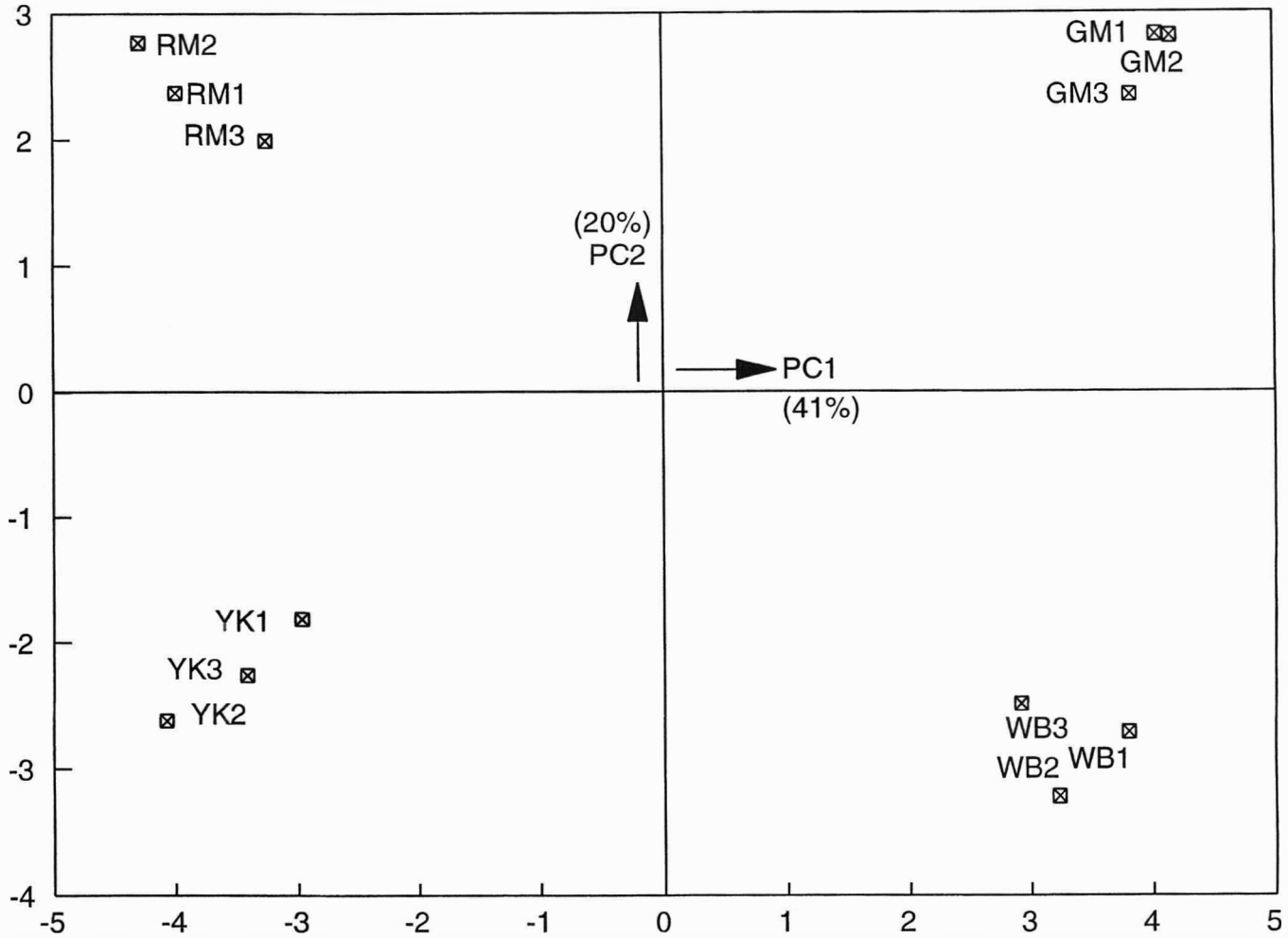

Fig. 4—PCA sample scores of hot-air dried green and red cv. Mazurka, white cv. Blondy, and yellow cv. Kelvin. (Codes, Table 4.)

Relationships between odor compounds and aroma attributes of fresh bell peppers

Relationships between the composition of volatile odor compounds and the evaluated aroma attributes of different colored fresh and processed bell pepper samples were studied by principal component analysis (PCA). Odor compounds and aroma attributes, which differed significantly between two or more colors were included in the data set. PCA on the data of the fresh fruits revealed three principal components (PC) accounting for 74% of variation. Sample scores on the first two PC's (Fig. 2) showed that the unripe green peppers were separated from the ripe yellow and ripe red peppers on PC1, whereas white fruits were separated from the others on PC2. The loadings plot (Fig. 3) demonstrates that on PC1 and PC2 several sensory aroma attributes and volatile odor compounds were related to each other.

On the negative side of PC1 aroma attributes herbal, grassy, green pepper, and resinous, and the volatile odor compounds (*Z*)-3-hexenol (19), hexanol (18), (*Z*)-3-hexenal (9), and hexanal (7) had relatively high loadings. The sniffing port panel characterized these compounds as grassy, green bell pepper, spicy, herbal, and lettuce-like (Table 5). Probably, these volatiles were responsible for corresponding "green" aroma attributes, which are typical for green fruits (Fig. 2). Similar relationships were observed for green fruits of cv. Evident (Luning et al., 1994c).

On the positive side of PC1 the aroma attribute fruity/chemical and several volatile odor compounds had high loadings. The volatiles 2-heptanone (10) and octanal (15) were closely related to this aroma attribute, and were described as fruity by the sniffing panel (Table 5). Possibly, they contributed to the perceived fruity/ chemical aroma, typical for both fresh red and yellow peppers (Fig. 2).

On PC2, red bell peppers had high positive scores (Fig. 2). The volatile compounds 13 and 21 and the aroma attributes spicy, rancid, sweet/sickly, musty, rubbery, sharp and hay had high positive loadings on PC2 as well (Fig. 3), and therefore are typical for red peppers. The odor compounds (*E*)-2-hexenal (13) and (*E*)-2-hexenol (21) and the aroma attributes spicy and sweet/sickly were relatively close and thus showed a relationship. The spicy, sweet, and almond odor notes of these volatiles may be important for corresponding aroma attributes. Additionally, several volatile compounds, located on the positive side of PC1, like 3-carene (8), *o*-xylene (11), (*Z*)-β-ocimene (14), 4-octen-3-one (16), and 6-methyl-5-hepten-2-one (17) had musty, earthy, sweaty, rancid, and rubbery odor characteristics. However, they showed no relationships with corresponding aroma attributes rubbery, musty and rancid/sweaty which had higher loadings on PC2 than on PC1 (Fig. 3).

On the negative side of PC2 (Fig. 3), the aroma attributes floral, cucumber, and fruity/fresh, and the odor compounds (*E*)-2-nonenal (24) and linalool (25) had high loadings, and were typical for white fruits (Fig. 2). Moreover, the cucumber and floral notes of the volatiles (Table 5) were similar to the closely located attributes. Examination of the third PC, which accounted for 11% of variance, revealed no further grouping of instrumental and sensory data. The red and yellow fruits were separated from each other on the presence of (*E*)-2-hexenol and (*E*)-2-hexenal; the compounds were typical for red fruits.

Partial least-squares regression (PLS) confirmed the three PC's obtained by PCA and showed similar relationships. PLS

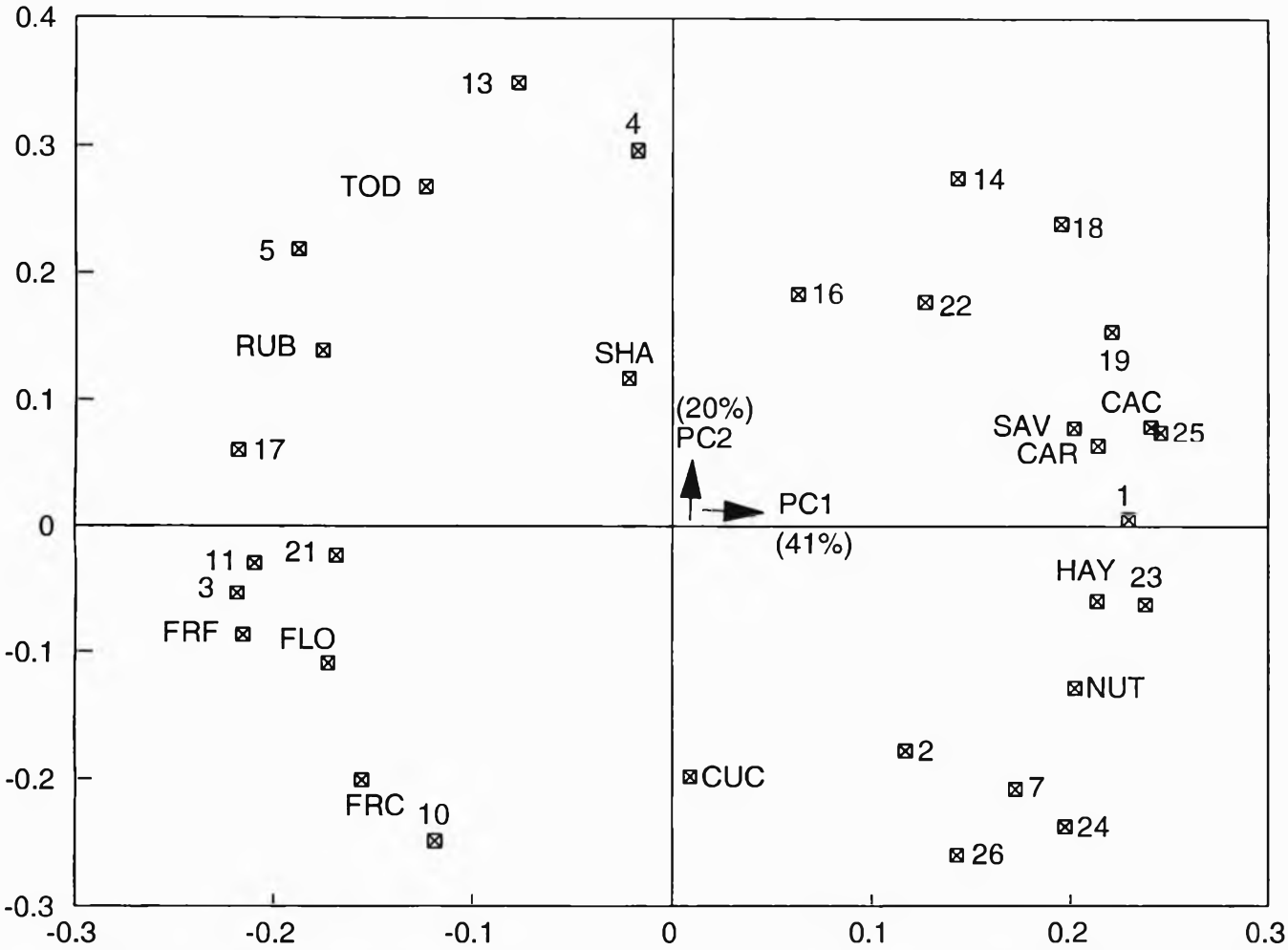

Fig. 5—PCA loadings of aroma attributes and percentage peak areas of odor compounds of hot-air dried peppers. (Codes, Table 4 and 5.)

revealed that 70.3% of variance in the X-data (instrumental) explained 66.8% of variance in the Y-data (sensory).

Relationships between odor compounds and aroma attributes of rehydrated hot-air dried peppers

PCA on the data set of the rehydrated dried fruits revealed three principal components (PC) accounting for 73% of variation. Sample scores on the first two PC's (Fig. 4) showed that the green and white peppers were separated from the red and yellow fruits on PC1. On PC2 the green and white, and the red and yellow samples were differentiated from each other. Distribution of aroma attributes and volatile odor compounds on PC1 and PC2 was more diffuse (Fig. 5) as compared to fresh samples.

On the negative side of PC1 the aroma attributes rubbery, fruity/fresh, and floral, and the odor compounds 6-methyl-5-hepten-2-one (17), *o*-xylene (11), (*E*)-2-hexenol (21), and 3-methylbutanal (3) had high loadings and are therefore typical for red and yellow rather than for white and green fruits. The volatile compound *o*-xylene (11) had a rubbery odor but it showed no strong relationship with the aroma attribute rubbery (Fig. 5). In fact, *o*-xylene (11) and 3-methylbutanal (3) were located near to the aroma attribute fruity/fresh but their odor notes, characterized by sniffing panel, were completely different. However, Holscher and Steinhart (1994) described 3-methylbutanal as fruity and pungent. Possibly, this compound contributed to the fruity impression of dried yellow and red peppers.

On the positive side of PC1 the aroma attributes savory, caramel, cacao, hay, and nutty, and the volatile odor compounds 2-methylpropanal (1), (*Z*)-3-hexenol (19), linalool (25), and 2-isobutyl-3-methoxypyrazine (23) had relatively high positive loadings. Therefore, they were typical for dried green and white peppers more than for yellow and red peppers. The green, spicy characteristics of volatiles 19 and 23, and the cacao, rancid/sweaty odor of 2-methylpropanal (1) support corresponding aroma attributes.

On PC2, only the aroma attribute fruity/chemical and the fruity smelling compound 2-heptanone (10) were relatively close to each other (Fig. 5). They were typical for dried yellow samples which had relatively high negative loadings on PC2 as well (Fig. 4). The dried tomato aroma was more typical for red samples since it was positively located on PC2. No odor compounds were related to this aroma attribute. Some volatile compounds like hexanal (7), (*E*)-2-nonenal (24), and (*E,Z*)-2,6-nonadienal (26) had high positive loadings on PC1 as well as high negative loadings on PC2. Therefore, these odor compounds were typical for both the dried green, white, and yellow samples as compared to red (Fig. 5). However, no aroma attributes were closely related to these odor compounds, indicating they were less important for differences in perceived aroma of dried fruits. On the third PC, which accounted for 12% of variance, no further grouping of instrumental and sensory data was observed.

PLS confirmed only the first two PC's obtained by PCA and showed similar relationships. PLS revealed that 68.3% of variance in X-data explained 46.2% of variance in Y-data.

In our study, relationships between aroma attributes and volatile odor compounds were less distinct in dried than in fresh peppers. Drying reduced the amount of odor compounds and

diminished differences in composition of volatiles between fruit colors (Tables 3 and 4). Consequently, it appeared to be more difficult to differentiate between aromas of different colored dried samples. This confirmed the conclusion of Van Ruth and Roozen (1994), who suggested that commercial Chilean, Turkish, and Hungarian dried peppers were more different in taste than in aroma attributes.

CONCLUSIONS

HOT-AIR DRYING greatly changed the composition of fresh bell peppers. The decrease of volatile compounds with fruity, green, vegetable-like, and floral odors seemed to be reflected in decreased intensity of corresponding aroma attributes after drying. The increase of volatile compounds with cacao, spicy, sweaty, rancid and sweet, caramel-like odor notes during drying, may be correlated with some typical aroma attributes of dried bell peppers like savory, rancid/sweaty, and sweet/sickly, and hay-like aroma.

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Ms received 2/6/95; revised 4/25/95; accepted 5/25/95.

This research was supported by the Danish Ministry of Education as part of the FØTEK programme and by The Danish Ministry of Agriculture and Fisheries.

Chilling-Associated Softening of Tomato Fruit is Related to Increased Pectinmethylesterase Activity

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ABSTRACT

Mature-green tomatoes chilled 15 days (5°C; RH >85%) were softer than nonchilled during subsequent ripening (22°C) by both whole fruit and pericarp tissue puncture ($p<0.05$), but not by flat-plate compression. No differences in total polygalacturonase (PG) or PG isozyme activity were evident although total activity was greater in nonchilled after 10 days ripening. Softening of nonchilled fruit correlated ($p<0.05$) with extracted PGI activity, while chilling-associated softening correlated ($p<0.05$) with higher initial extracted pectinmethylesterase (PME) activity. Extracted peroxidase remained constant throughout ripening but was greater ($p<0.05$) in pre-chilled fruit consistent with chill-induced membrane dysfunction. Transmission electron microscopy showed the middle lamella from pre-chilled tomatoes was swollen and less defined. Loss of turgor from translocation of water to the PME-modified cell wall was suggested to be responsible for softening as a consequence of chilling.

Key Words: tomato, ripening, softening, pectinesterase, texture

INTRODUCTION

CHILLING INJURY (CI) is the physiological damage in many plants and plant products incurred upon exposure to low but nonfreezing temperatures (Raison and Lyons, 1986; Parkin et al., 1989). The tomato is a chill-sensitive fruit in which chilling response may be classified according to extent of damage (Hobson, 1987): slight CI, loss of firmness and nonuniform coloration; moderate CI, translucent water-soaked patches and/or mottled yellow areas on red fruit and uneven surface; and severe CI, large, firm, extensive green patches on red fruit, uneven surface contours, desiccation and wrinkling. CI symptoms that often become apparent only after removal of fruit from chilling temperatures can lead to poor overall quality (Morris, 1982; Jackman et al., 1988).

Among the many attributes that contribute to tomato quality, appearance and firmness may be the most important (Davies and Hobson, 1981). Progressive loss of firmness, or softening, of tomatoes as a consequence of normal ripening is complex, involving many different mechanisms (Sakurai and Nevins, 1993; Jackman and Stanley, 1995). There is loss of cell turgor and non-enzymatically mediated reduction in molecular weight-size distribution of cell wall polymers. Also, the action of cellulase, pectinmethylesterase (PME; EC 3.1.1.11) and various other cell wall glycan hydrolases contribute to ripening-associated softening (Hobson, 1968; Wallner and Walker, 1975; Huber, 1983a,b; Fishman et al., 1989; Mitcham et al., 1989). However, little correlation has been reported between such other enzymatic and nonenzymatic mechanisms and the rate of fruit softening. Although peroxidase (POX; EC 1.11.1.7) is not directly involved in softening, an increase in its activity and its ability to catalyze auxin degradation may be required before ripening changes occur (Thomas et al., 1981). High levels of this enzyme are often associated with oxidative deterioration in many plants that are in advanced stages of ripening or senescence.

Although still uncertain [see reviews by Gross (1990) and Hobson and Grierson (1993)], tomato fruit softening has been primarily attributed to depolymerization and solubilization of polyuronides in the middle lamellae of cell walls by endo-polygalacturonase (PG: poly-1,4-*D*-galacturonide glycanhydrolase, EC 3.2.1.15) (Brady et al., 1982; Huber, 1983a; DellaPenna et al., 1990). Levels of PG activity generally increase as ripening progresses and are greater in fruit exhibiting increased softness. Unexpectedly, mature-green tomato fruit with symptoms of slight to moderate CI are often softer than nonchilled tomatoes of the same physiological age, although PG activity is not detectable (Jackman and Stanley, 1992; Jackman et al., 1992a). Previous results have suggested that the enhanced softening of chill-injured tomato fruit may be related to physicochemical changes that occur during chilled storage, that may also lead to development of mealiness (Marangoni et al., 1989a; Jackman et al., 1992a,b). A related disorder in chilled peaches and nectarines (woolliness) has been largely attributed to activity of PME (Dawson et al., 1992; 1993; Harker and Sutherland, 1993). The enzymatic de-esterification of cell wall pectin is not totally abated by chilling temperatures. Thus sites for absorption of water and consequent swelling of the middle lamellae, and the amount of substrate available for action of PG (subsequently induced by warmer temperatures), increase during chilled storage. Similar changes to cell wall pectin in tomatoes may be manifest by increased softening.

Our objective was to monitor the activities of total PG, PGI and PGII isozymes, PME and POX in chilled (5°C, 85% RH, 15 days) and nonchilled mature-green tomato fruit over a 10 day ripening period, to investigate whether changes in activity of the enzymes correlated with firmness of fruit.

MATERIALS AND METHODS

UNLESS OTHERWISE INDICATED, all chemicals and reagents used in this investigation were purchased from Sigma (St. Louis, MO) and were reagent grade.

Plant material

Mature-green tomato fruit (*Lycopersicon esculentum* cv. Caruso) at ripening stages 3 and 4 (Kader and Morris, 1976), *i.e.* locule contents had developed some pink pigmentation and become somewhat liquid, were harvested at random from 50 different plants in a commercial greenhouse (Costa Greenhouse, Paris, ON). Two fruit were collected from each plant. The fruit were surface sterilized by immersion in 2.0% (v/v) commercial bleach (5% sodium hypchlorite solution) for 3 min, followed by rinsing in deionized water for 3 min. Fifty fruit were stored at 5°C (85% RH) for 15 days, then transferred to 22°C for an additional 10 days to facilitate ripening. Fifty "control" fruit were immediately allowed to ripen at 22°C for 10 days without pre-chilling. Five tomatoes from each of the pre-chilled and nonchilled storage treatments were randomly selected at day 0, 2, 5, and 10 of ripening for each of texture evaluation and enzyme assay.

Firmness evaluation

Force-deformation profiles were recorded using a "M" Series Materials Testing Machine (J.J. Lloyd Instruments, Omnitronix Ltd., Mississauga, ON) as described by Jackman et al. (1990). The maximum force (F_m) and deformation incurred by that force (L_m) were determined from the profiles and used to calculate firmness (F_m/L_m).

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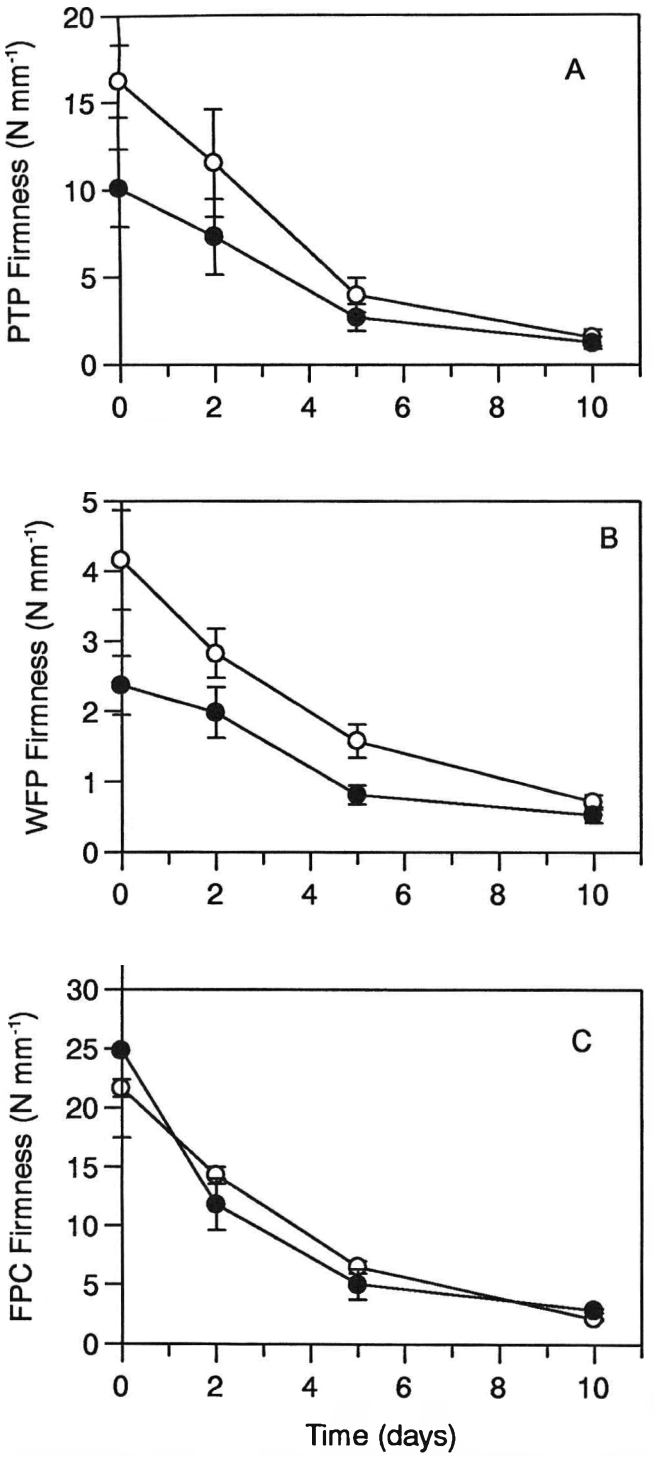

Fig. 1—Changes in firmness of pre-chilled (●) and nonchilled (○) tomatoes over a 10-day ripening period, as measured by (A) pericarp tissue (PTP) and (B) whole fruit (WFP) puncture (PTP), and (C) flat-plate compression (FPC). Each point represents the mean of five determinations; bars are standard deviations.

Flat-plate compression (FPC). Whole tomato fruit were placed on a stationary steel plate aligned from stem end to stylar end, with the stylar end facing down, and compressed with a 17.8 × 11.7-cm upper steel plate, in conjunction with a 5-kN load cell, at a rate of 10 mm min^{-1}.

Whole fruit puncture (WFP). Whole tomato fruit were placed in a plastic holder to prevent slippage during puncture tests. Each fruit was punctured at three separate locations along the equatorial axis with a 1.6-mm flat-ended, cylindrical probe, in conjunction with a 50-N load cell, at a rate of 5 mm min^{-1}.

Pericarp tissue puncture (PTP). Three 1.5 × 1.5-cm pericarp samples were excised from the equatorial region of each tomato fruit, carefully avoiding regions where the columellar tissue joined the pericarp. Pericarp samples were placed exocarp down on a steel plate and punctured at a rate of 5 mm min^{-1} with an 11-mm flat-ended cylindrical probe in conjunction with a 500-N load cell.

Enzyme assay

Tissue homogenates. Two 100-g composite samples of sliced pericarp tissue from the five different tomatoes used in texture evaluation were each homogenized at 4°C for two successive 1 min intervals in 100-mL of 0.02-M MES [2-(N-morpholino)ethanesulfonic acid] buffer (pH 6.0) containing 1.2-M NaCl, 0.5% (w/v) insoluble polyvinylpyrrolidone and 1-mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 10,000 × *g* for 30 min, then filtered through Miracloth (Calbiochem-Behring Corp., LaJolla, CA). The resulting filtrate was either assayed directly for PG, PME and POX activities, or immediately frozen at -18°C. Frozen samples were assayed within 1 wk. Protein content of each extract was determined in triplicate by the method of Bradford (1976), using defatted bovine serum albumin as reference. The values reported are means of the two extracts.

Polygalacturonase (PG). Total PG activity was measured by adding 0.1-mL of filtered extract to 0.2-mL of 0.1M sodium acetate (pH 4.5) and 0.2-mL of 0.15M NaCl. Blanks were prepared by heating duplicate samples 5 min at 100°C. Reactions were initiated by adding 0.5-mL of 1% (w/v) polygalacturonic acid (Sigma Grade III) adjusted to pH 4.5 (Pressey, 1985). After 30 min at 37°C, reaction mixtures were analyzed for reducing groups by the arsenomolybdate method of Nelson (1944). Activity of the PGI isozyme (Ali and Brady, 1982) was estimated by measuring residual activity in reaction mixtures prepared as above, but heated 5 min at 65°C before addition of substrate (Tucker et al., 1980). PGII activity was then estimated from the difference between total PG and PGI activities. PG activity was expressed as nmole galacturonic acid reducing group equivalents min^{-1} mg $protein^{-1}$.

Pectinmethylesterase (PME). PME was assayed by the spectrophotometric procedure of Hagerman and Austin (1986). Two milliliters of 0.5% (w/v) citrus pectin (pH 7.5) were combined in a cuvette with 0.15-mL of 0.01% (w/v) bromothymol blue in 0.003M potassium phosphate buffer (pH 7.5), and 0.83-mL of distilled water adjusted to pH 7.5. After adding 20-µL of filtered homogenate, the rate of decrease in absorbance at 620 nm *vs* a water blank was recorded for 1 min on a UV/Visible Recording Spectrophotometer (Model UV-260, Shimadzu Corporation, Kyoto, Japan). PME activity was expressed as µmole galacturonic acid equivalents min^{-1} mg $protein^{-1}$.

Peroxidase (POX). POX activity was assayed at 25°C by adding 0.3-mL of filtered homogenate to 2.7-mL of reaction mixture [100-mL of 0.1M citrate buffer (pH 6.2), 10-mL of 1% (v/v) guaiacol in 50% ethanol, and 10-mL of 0.3% H_2O_2]. Absorbance at 470 nm was recorded for 1 min *vs* a reagent blank (Chance and Maehly, 1955). POX activity was expressed as change in absorbance min^{-1} mg $protein^{-1}$.

Transmission electron microscopy (TEM)

Sample preparation and TEM of pericarp tissue during ripening of pre-chilled and nonchilled tomato fruit was as described by Marangoni et al. (1989b).

Statistical analysis

Each of the five tomatoes from each storage treatment × time combination represented a replicate in analysis of variance (ANOVA) of texture data. Triplicate enzyme assays were performed for each of two replicate tomato extracts from each storage treatment × time combination. ANOVA and correlation analysis were performed using the Statistical Analysis System package (SAS Institute Inc., 1985).

RESULTS & DISCUSSION

Tomato firmness

Firmness of pre-chilled and nonchilled tomatoes and pericarp tissues progressively decreased ($p<0.01$) over the 10 day ripening period (Fig. 1). The puncture (WFP and PTP) firmness of pre-chilled fruit and tissue was lower ($p<0.05$) than nonchilled tomato fruit and tissue at day 0, but approached that of nonchilled fruit and tissue over 10 days ripening at 22°C (Fig. 1A and 1B). A greater rate of softening was observed for tissue *vs* whole fruit for both pre-chilled and nonchilled tomatoes. Chilling-associated softening has been considered symptomatic of

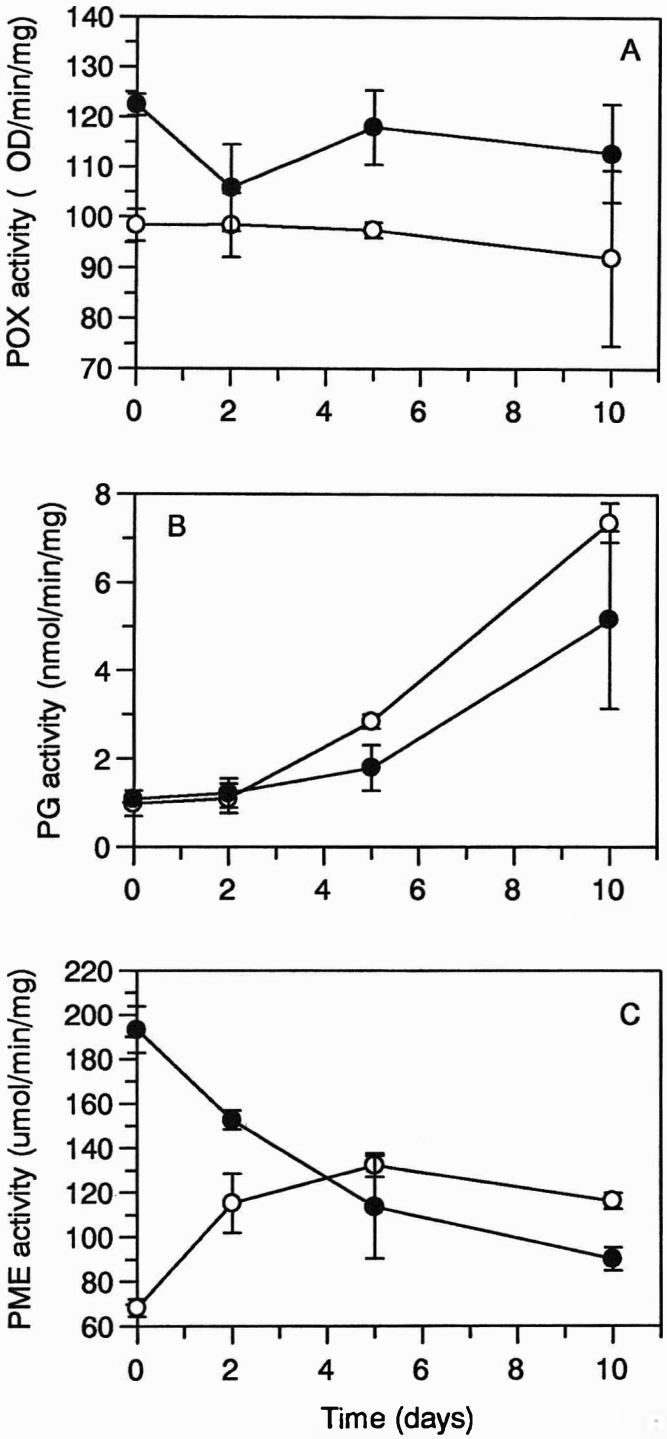

Fig. 2—Changes in activity of (A) extracted peroxidase (POX), (B) total polygalacturonase (PG) and (C) pectinmethylesterase (PME) from pre-chilled (●) and nonchilled (○) tomatoes during a 10-day ripening period. Each point represents the mean of two replicate determinations.

slight to moderate CI in tomato fruit (Hobson, 1987; Jackman and Stanley, 1992; Jackman et al., 1992a). Confirming previous reports (*cf.*, Jackman et al., 1992a), a decreased rate of red color development and uneven, blotchy ripening were also observed for chilled fruit (data not shown).

In contrast to results from WFP and PTP tests, pre-chilled and nonchilled tomatoes showed no differences in initial FPC firmness or rate of softening during 10 days ripening (Fig. 1C), as reported previously (Jackman et al., 1990; 1992a). Thus, the FPC test may be less sensitive than puncture tests in differentiating tomato fruit that differ in firmness. The FPC test is one of the most common methods used to measure tomato firmness. Giovannoni et al. (1989), on the basis of compression tests, reported that PG was the primary determinant of cell wall polyuronide degradation, but that the degradation alone did not account for tomato fruit softening. Similarly, compressibility of transgenic fruit expressing antisense PG RNA, and substantially reduced PG activity did not differ from control wild-type fruit (Smith et al., 1988; Schuch et al., 1991; Kramer et al., 1992). Firmness values derived from compression tests are based on the properties of the whole tomato and depend, among other things, on the strength and toughness of skin, integrity of pericarp, number of locule walls, size of fruit and overall turgor (Holt, 1970). In contrast, puncture tests may better reflect changes in the integrity of pericarp in which cell wall hydrolases (*e.g.*, PG) are localized (Tieman and Handa, 1989). They would thus seem to be more appropriate than FPC for measurement of firmness (*cf.* Jackman et al., 1990).

Enzyme activity

The activity of POX extracted from pre-chilled and nonchilled tomatoes did not change ($p<0.05$) over 10 days of ripening; however, greater levels were obtained from pre-chilled fruit (Fig. 2A). Thomas et al. (1981) reported that soluble and ionically bound peroxidase activities increased sharply during early stages of normal tomato development, reaching a maximum at the mature green stage and slowly decreasing thereafter. The level of POX activity in tomatoes may be indicative of the susceptibility of fruits to ripening-associated changes (Thomas et al., 1981), since it reflects a greater oxidation status of the fruit/tissue. Decompartmentation, as a consequence of senescent-like changes during chilling (*cf.* Parkin et al., 1989; Palma et al., 1995), may have led to the increase in extractable POX activity. The microstructure of nonchilled fruit contrasted with that of pre-chilled fruit, for which disruption of chloroplast thylakoids, clumping of plastoglobuli and general loss of subcellular contrast and structure, particularly that of membranes, was evident (Fig. 3). This general degradation of subcellular membranes may have been due to lipid peroxidation, possibly mediated by POX and characteristic of senescent-like processes (Paliyath and Droillard, 1992). The activity of extracted POX was not correlated with softening of pre-chilled or nonchilled tomatoes (Table 1).

The activity of total extracted PG (Fig. 2B), and of PG isozymes (not shown), increased ($p<0.01$) with ripening, in agreement with other results (Tucker et al., 1980; Brady et al., 1982; Tucker and Grierson, 1982). The enzyme initially occurred predominantly as PGI (*cf.* Pogson et al., 1991), and extracted levels of this isoform remained relatively constant throughout ripening. However, after 10 days at 22°C levels of extracted PGI activity accounted only for about 25% of the total PG activity indicated in Fig. 2B. Brady et al. (1987) suggested that although PGI was distributed in a nonrandom, localized manner and was cell wall bound, that which accumulated early in ripening could rapidly and extensively depolymerize polyuronides in the wall. As indicated (Fig. 1), most tomato fruit softening occurs in the first week of ripening from the mature-green stage (*cf.* Jackman et al., 1990; 1992a) when PG is mainly in the form of PGI. Extracted PGI activity correlated ($p<0.05$) with firmness of nonchilled but not pre-chilled fruit (Table 1). This supported general inverse correlations previously noted between PG activity and fruit firmness (Hobson, 1965; Ahrens and Huber, 1990), and between activity of PGI and fruit firmness (Brady et al., 1983, 1985), during different stages of "normal" tomato fruit ripening. The pre-chilling treatment had no significant influence on the total extracted activity of PG, or that of the PG isozymes, although extracted levels were generally lower in pre-chilled fruit as ripening progressed (Fig. 2B).

Chilling of mature-green tomatoes at 5°C (RH >85%) for 15 days prior to transfer to 22°C for subsequent ripening led to greater ($p<0.05$) initial levels of extracted PME compared to those from nonchilled fruit (Fig. 2C). By day 2 of ripening, and

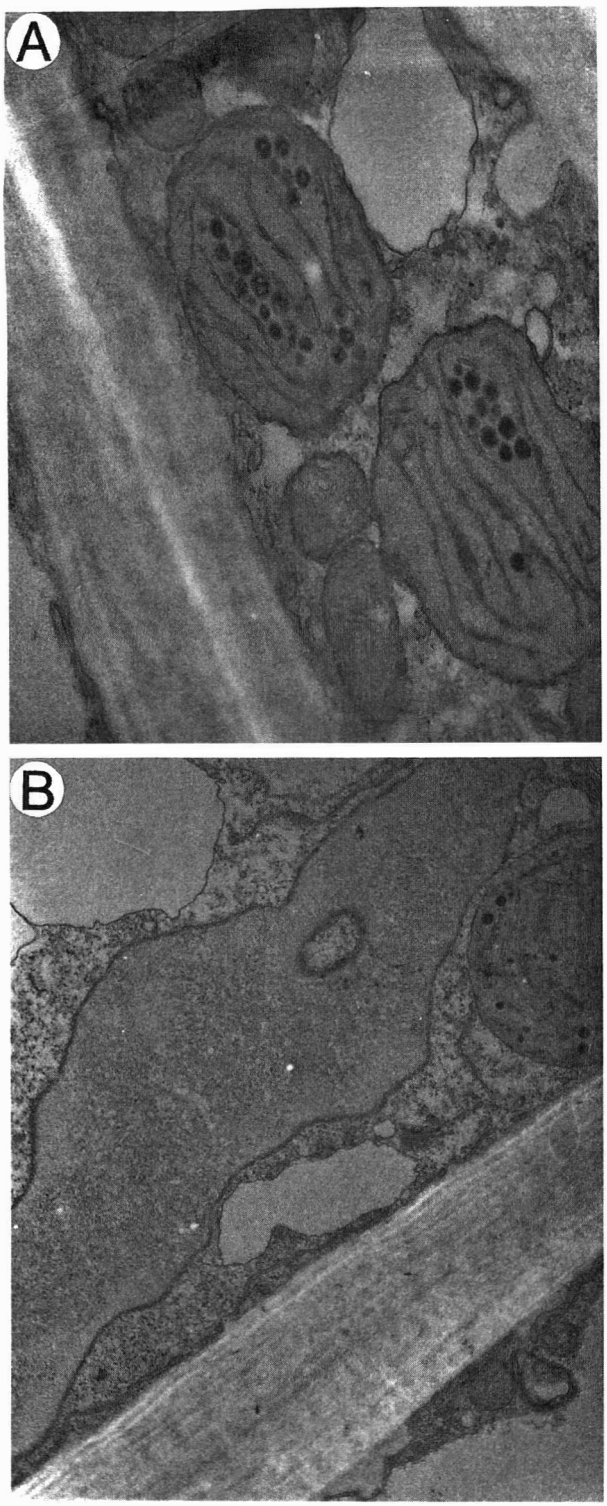

Fig. 3—Transmission electron micrographs of (A) pre-chilled tomato fruit pericarp cells and (B) pericarp tissue cells from nonchilled tomatoes ripened at 22°C for 8 days. (A) The pre-chilled cells show alterations in the middle lamella, shown here as a white zone. Chloroplast thylakoids have unstacked and general subcellular features, particularly membranes, seem compromised (×31,100). (B) The ripened cells do not display the alteration in middle lamella structure and general subcellular features are clearly preserved (×18,600).

thereafter, extracted PME from pre-chilled and nonchilled tomatoes did not differ. Firmness of pre-chilled tomatoes correlated ($p<0.05$) with activity of extracted PME, but not with total PG or PG isozymes (Table 1). Similar changes in pectolytic enzyme activity as in the pre-chilled tomato fruit have been

Table 1—Correlation of activity of total polygalacturonase (PG), PGI and PGII isoenzymes, pectinesterase (PME) and peroxidase (POX) with flat-plate compression (FPC), and whole fruit and pericarp tissue puncture (WFP and PTP) firmness of pre-chilled (5°C; RH >85%; 15 days) and non-chilled mature green tomato fruit during a 10 day ripening period (n=4)

Treatment	Enzyme Activity	Firmness		
		FPC	WFP	PTP
Nonchilled	PG	0.851	−0.858	−0.839
	PGI	−0.957*	−0.949	−0.976*
	PGII	−0.782	−0.794	−0.762
	PME	−0.812	−0.809	−0.802
	POX	0.776	0.787	0.757
Chilled	PG	−0.724	−0.787	−0.761
	PGI	−0.741	−0.823	−0.786
	PGII	−0.701	−0.748	−0.731
	PME	0.994**	0.981*	0.995**
	POX	0.322	0.120	0.235

*$p<0.05$; **$p<0.01$.

deemed responsible for woolliness in chilled peaches and nectarines (Pressey and Avants, 1978; Ben-Arie and Sonego, 1980; Von Mollendorff and De Villiers, 1988). In those fruit the development of woolliness, a physiological disorder similar to mealiness, resulted from altered pectic polymer breakdown and a consequent increase in water absorption by cell walls (Dawson et al., 1992; 1993; Harker and Sutherland, 1993). Consistent with those observations, tomatoes chilled for extended periods exhibited reduced ion leakage upon removal of fruit to warmer temperatures, rather than expected increases (Cote et al., 1993). The effect was due to an increase in pectin demethylation during chilling (Jackman et al., 1992a) which increased the number and availability of carboxyl groups for binding with calcium and other ions, in addition to water, thereby reducing the amount of measured ion leakage. The softening of fruit and tissue that occurred during chilling was not likely due to de-esterification of cell wall polyuronides per se, especially if divalent cations such as calcium were available (Huber, 1983a).

A change in structure or dissolution of middle lamellae, manifest by regions of reduced electron density, was evident for mature-green tomato fruit that had been previously chilled (5°C; RH >80%) for 15 days (Fig. 3A). Similar observations were reported by Marangoni et al. 1989a), and suggest the involvement of PME. The regions of reduced electron density could be attributed to water absorption by the cell wall, mediated by PME-alteration of middle-lamellar pectin. Translocation of water from the cytosol would readily occur with concomitant increases in availability of binding or hydration sites in the wall and chill-induced membrane dysfunction (Jackman et al., 1992a,b). Transpirational losses during chilling (*cf.* Jackman et al., 1992a) would contribute also to overall loss of turgor. The resulting turgor loss, combined with increased water absorption or swelling of cell walls, may be responsible for chilling-associated softening. These conclusions are consistent with previous results with tomatoes (Jackman and Stanley, 1992; Jackman et al., 1992a,b) and with mechanisms of woolliness development in chilled peaches and nectarines (Dawson et al., 1992; 1993; Harker and Sutherland, 1993).

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Ms received 3/13/95; revised 5/30/95; accepted 6/17/95.

The authors acknowledge the financial assistance of the Natural Sciences & Engineering Research Council of Canada and the Ontario Ministry of Agriculture & Food.

Ascorbic Acid Stability in Ground Asparagus Samples and in Oxalic Acid Extracts

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ABSTRACT

To establish the storage conditions for asparagus preparation before ascorbic acid determination, samples were ground, the mass was divided into three aliquots that were, respectively, stored at 4°C, −18°C, or extracted with 1% oxalic acid. The extract was further split into aliquots and stored at 25°, 4°, −18° and −75°C. Ascorbic acid content was measured at different times of storage by a polarographic method. The rate of degradation increased with storage temperature; the degradation rate was higher in ground samples than in extracts; no significant changes in ascorbic acid content were observed in extracts stored for 7 days at −18° or for 90 days at −75°C.

Key Words: asparagus, ascorbic acid, oxalic acid, storage stability

INTRODUCTION

ASCORBIC ACID (AA) oxidizes in three steps. Initially a reversible reaction yields dehydroascorbic acid, which by hydrolysis gives 2,3-diketogulonic acid, which can oxidize to treonic and oxalic acids, thus losing totally and irreversibly all vitamin activity. When ascorbic acid is measured in biological samples several precautions during sampling, treatment and extraction of the sample must be taken to prevent changes in ascorbic acid content. To prepare stable ascorbic acid solutions air and oxygen should be excluded, the pH must be low, metallic contamination must be avoided and the sample must be protected from direct light (Blaug and Hajratwala, 1972; Hurt, 1979; Leon et al., 1982; Nobile and Wodhill, 1981).

The stability of AA is higher in an acid medium. Thus, stabilization of AA and extraction efficiency by several acids have been studied (Archer, 1981; Freebairn, 1959; Johnsson and Hessel, 1982). Metaphosphoric acid, first reported by Fujita and Itwataka (1935) was an efficient extractant that also inactivated the catalytic effect of ascorbate oxidase. Copper, precipitates proteins and favors the elimination of enzymatic oxidases and the extract clarification.

Others (Ponting, 1943; Shumin et al., 1983) have pointed out the suitability of metaphosphoric and oxalic acids for extracting ascorbic acid from biological samples. The official AOAC fluorometric method of ascorbic and dehydroascorbic acid determination uses a metaphosphoric:acetic acid mixture (AOAC, 1990). The main disadvantage to the use of metaphosphoric acid is its instability. For this reason several alternatives have been proposed, including 0.05M perchloric acid or 3% trichloroacetic acid alone (Freebairn, 1959; Kim et al., 1987) or in mixture with 0.05M perchloric acid. To prevent the catalytic action of metals the use of ethylene diaminetetraacetic acid has been proposed (Jager, 1948).

Because of the instability and cost of metaphosphoric acid (1%) an aqueous solution of oxalic acid was used as extractant of ascorbic acid of asparagus in preparation of a polarographic determination method (Esteve et al., 1995). No problems in ascorbic acid stability were detected, when the measuring was done a short time after sampling. When this is not possible because of the large number of samples to analyze, the samples should be stored in appropriate conditions to prevent ascorbic acid loss.

The objective of our study was to select the optimal conditions for storing samples until the time of analysis.

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MATERIALS & METHODS

ASCORBIC ACID was determined by applying the polarographic method proposed by Esteve et al. (1995). Assessment of the analytical parameters:

—Precision (relative standard deviation): Instrumental = 2.77%; Method = 4.0%.

—Accuracy measured by recovery assays: 96.9%.

Sampling

Bunches of 500g asparagus were used. From each bunch 100g were taken and ground in a nitrogen atmosphere. The ground mass was divided into three aliquots: r—stored in a refrigerator at 4°C; f—kept in a freezer at −18°C (in 2g portions); e—extracted with oxalic acid 1%. The extract was split into 1-mL aliquots, which were stored at 25°, 4°, −18°, and −25°C.

RESULTS & DISCUSSION

IN ORDER TO ESTIMATE AA stability the mean contents of aliquots stored in different conditions were measured at different times of storage. To find the pattern of relationships between AA content and storage time (days), the equation and correlation coefficients between AA content (x) and time (y) were calculated. The ANOVA of a controlled factor time was applied to these values to detect significant differences and then the Tukey test was applied to ascertain what values differed. AA content (mg/100g) of ground sample stored at 4° and −18°C at different times was summarized (Table 1). Losses began to be statistically significant the day after treatment. Ascorbic acid losses in oxalic acid extract stored at 25°C (Table 2) began to be statistically significant 4 hr after extraction. The decrease was gradual, after the first 3.5 hr.

The results for the oxalic acid extract stored under refrigeration (4°C) or frozen (−18 or −75°C) were compared (Table 3). At 4°C ascorbic acid losses began to be significant on the second day of storage, and from that time a gradual decrease was observed. At −18°C AA losses began to be statistically significant on the 14th day. Ascorbic acid content of the oxalic

Table 1—Ascorbic acid stability in ground asparagus sample

Time (days)	Content (mg/100g)	
	4°C	−18°C
0	11.39 ± 0.52[a]	9.13 ± 10.25[a]
1	8.56 ± 0.03[b]	8.17 ± 0.33[ab]
3	7.22 ± 0.54[c]	7.91 ± 0.77[abc]
6	3.89 ± 0.14[d]	7.21 ± 0.24[bc]
10	—	6.29 ± 0.33[c]
Equation[e]	y = 10.632 − 1.147x	y = 8.759 − 0.254x
Correlation coefficient	0.975**	0.971**

[a-d] No coincidence in letters indicates significant differences.

[e] y = time (days); x = ascorbic acid content.

** Significant $p<0.01$.

Table 2—Ascorbic acid stability in oxalic acid extract kept at 25°C

Time (hr)	Content (mg/100g)
0	11.39 ± 0.52[a]
0.5	11.20 ± 0.17[a]
1	11.07 ± 0.07[a]
1.5	10.33 ± 0.28[a]
2	10.59 ± 0.74[a]
3	10.45 ± 0.83[ab]
3.5	10.33 ± 0.28[abc]
4	8.88 ± 0.49[bc]
4.5	8.78 ± 0.14[c]
5	7.07 ± 0.54[d]
Equation[e]	y = 11.817 − 17.139x
Correlation coefficient	0.905**

[a-d] No coincidence in letters indicates significant differences.
[e] y = time (days); x = ascorbic acid content.
** Significant $p<0.01$.

extract stored at −75°C remained constant, with no losses in relation to initial concentration detected during the storage period. There ascorbic acid degradation was not linear. This could be explained by the fact that the slope was near zero, with a very low degradation rate.

CONCLUSIONS

COMPARISON OF SLOPES of ascorbic acid degradation curves corresponding to aliquots kept in different storage conditions showed that they were not parallel. Therefore, the rate of degradation was different in each of the assayed conditions. As expected, degradation rate increased with storage temperature. Also the degradation rate was higher in ground samples than in the oxalic acid extract. The most stable form of storage was the oxalic acid extract. No significant changes was observed in the first 7 days or in 90 days when stored at −18 and −75°C, respectively.

Table 3—Ascorbic acid stability in oxalic acid extracts stored at 4, −18 and −75°C

Time (days)	Content (mg/100g)		
	4°C	−18°C	−75°C
0	10.98 ± 0.23[a]	11.39 ± 0.52[a]	20.82 ± 2.24[a]
1	10.18 ± 0.25[a]	11.47 ± 0.85[a]	21.89 ± 0.13[a]
2	8.80 ± 0.80[b]	—	—
3	9.04 ± 0.10[b]	10.60 ± 2.06[ab]	22.08 ± 0.41[a]
6	6.14 ± 0.50[c]	—	22.89 ± 0.31[a]
7	6.01 ± 0.79[cd]	10.84 ± 0.66[ab]	—
8	4.95 ± 0.21[d]	—	17.58 ± 1.63[a]
14	—	9.33 ± 0.45[bc]	17.33 ± 0.27[a]
15	—	9.28 ± 0.11[bc]	—
20	—	9.32 ± 0.27[bc]	18.53 ± 0.27[a]
24	—	—	19.37 ± 2.88[a]
27	—	8.49 ± 0.04[c]	20.40 ± 0.36[a]
31	—	7.83 ± 0.29[cd]	20.30 ± 0.36[a]
38	—	6.43 ± 0.01[d]	—
42	—	—	22.36 ± 1.02[a]
50	—	—	19.19 ± 0.39[a]
68	—	—	21.30 ± 1.07[a]
90	—	—	21.84 ± 1.26[a]
Equation[e]	y=10.823−0.728x	y=11.372−0.120x	y=20.142+0.010x
Correlation coefficient	0.986**	0.980**	0.153 NS

[a-d] No coincidence in letters indicates significant differences.
[e] y = time (days); x = ascorbic acid content.
** Significant $p<0.01$.

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Ms received 3/2/95; revised 4/30/95; accepted 5/25/95.

Limonoids in Pummelos [*Citrus grandis* (L.) Osbeck]

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ABSTRACT

Juice and seeds from 16 cultivars of mature pummelo fruits were analyzed for limonoid content. Pummelo juice contained an average of 18 ppm limonin and 29 ppm total limonoid glucosides. Compared to other juices, pummelo contained very high concentrations of limonin and very low concentrations of limonoid glucosides. Limonin, nomilin, obacunone and trace amounts of deacetylnomilin were found in pummelo seeds. The 17-β-D-glucopyranoside derivatives (glucosides) of nomilin, nomilinic acid and obacunone were also present. Total limonoid aglycone concentration in the seeds ranged from 773 ppm to 9,900 ppm and total limonoid glucosides ranged from 130 ppm to 1,912 ppm.

Key Words: pummelos, citrus, limonin, limonoids, bitter principle

INTRODUCTION

LIMONOIDS are a group of chemically related triterpene derivatives found in the Rutaceae and Meliaceae families. There are two groups of limonoids in mature citrus fruit: the aglycones and corresponding glucosides. Among 36 aglycones and 20 glucosides isolated from citrus (Hasegawa and Herman, 1992), limonin is the major cause of delayed bitterness in juices of several major commercial citrus crops. The intact fruit do not normally contain limonin but rather a nonbitter precursor, limonoate A-ring lactone (LARL). LARL is gradually converted to limonin in the juice (Maier and Margileth, 1969). The degree of delayed bitterness, therefore, depends on LARL concentration in the fruit.

Pummelo, also known as Shaddock or Buntan, is widely grown in China and south east Asia. Because pummelos can be readily hybridized, there are very many distinct cultivars. There are large variations among them in fruit size, shape, rind thickness and internal colors. Our objective was to determine the limonoid content in the juices and seeds of 16 different cultivars.

MATERIALS & METHODS

Materials

Samples were collected on March 23, 1993 from the citrus varietal collection at the Citrus Research Center and Agricultural Experiment Station, Univ. of California, Riverside. A minimum of 12 fruits were collected from each cultivar.

For HPLC analysis, a Waters 6000A-510 pump system and a Perkin-Elmer (Norwalk, CT) LC-75 spectrophotometric detector were utilized. The HPLC column was a C-18 reversed-phase, Spherisorb ODS-2.5 μm (250 × 4.6 mm) column (Alltech Inc., Deerfield, IL). Silica gel HLF plates (Analtech, Newark, DE) were used for TLC analysis. C-18 Sep-Paks were purchased from Millipore Corp. (Milford, MA). Limonoid standards used had been isolated from citrus and characterized by NMR spectrometry.

Preparation of samples

Fruit from each group of pummelos were peeled and hand squeezed, and seeds were recovered. Juice samples (1 mL each), after 2 days in a refrigerator, were passed through a C-18 Sep-Pak, washed with H_2O, and eluted with MeOH. The MeOH fraction was used for both aglycone and glucoside analyses. Seeds were ground using a Brinkmann Retsch mill with 1 mm diameter opening sieves. Seed meal was first washed with hexane in a Soxhlet extractor to remove oily materials. The meal was then extracted with MeOH. The MeOH fraction was evaporated to dryness, re-dissolved in H_2O, and acidified to pH 3.0. Acidification converted the open D-ring monolactones to closed D-ring dilactones. The acidic aqueous fraction was then extracted three times with CH_2Cl_2. The combined CH_2Cl_2 fraction was evaporated to dryness and redissolved in CH_3CN for limonoid aglycone analysis. One mL of the remaining aqueous fraction was passed through a C-18 Sep-Pak cartridge, washed with 2 mL H_2O, and eluted with MeOH. This fraction was used for limonoid glucoside analysis.

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Limonoid aglycones analysis

Aglycones were identified by TLC on silica gel plates developed in three solvent systems: EtOAc-cyclohexane (3:2); EtOAc-CH_2Cl_2 (2:3); and CH_2Cl_2-MeOH (97:3). After drying, the plates were sprayed with Ehrlich's reagent and exposed to HCl gas (Dreyer 1965), and limonoid aglycones were identified by visual comparison with standards. HPLC was used to quantify aglycones (Ozaki et al. 1991). A small known amount of sample was evaporated to dryness and dissolved in mobile phase for injection. An isocratic system with CH_3CN-MeOH-H_2O (10:41:49) was used at 1 mL/min. Retention times for limonin, nomilin, and obacunone were 15, 26, and 45 min, respectively.

Limonoid glucosides analysis

The limonoid glucosides were analyzed by reverse-phase HPLC. A linear gradient system starting with 15% CH_3CN in 3 mM H_3PO_4 and ending with 27% CH_3CN over 36 min was used with flow rate 1 mL/min. Retention times for the glucosides of limonin, nomilin, nomilinic acid, and obacunone were 18, 29, 30, and 34 min, respectively (Herman et al., 1990).

RESULTS & DISCUSSION

MATURE FRUIT of 16 cultivars of pummelo were collected in late-season (March, 1993) and the juices and seeds were analyzed for limonoid content. Juices contained very high concentrations of limonin, with an average of 18 ppm for the 16 cultivars (Table 1). These results clearly show why pummelo

Table 1—Limonin and total limonoid glucoside in pummelo juices

Group	Cultivar	CRC #[a]	pH	Limonin[b]	TG[c]
A	African	2346	3.70	32.5	63
B	Arajon	2596	3.45	14.0	30
C	Deep Red	2347	3.69	25.0	23
D	Kao Phuang	2352	3.61	13.5	17
E	Kao Ruan Tia	2351	3.40	10.0	45
F	Nakon Chaisi	2353	3.55	4.0	10
G	Philippine	644	3.95	17.5	7
H	Pin Shan Kong Yau	2348	3.53	14.5	13
I	Pink	2244	3.27	20.0	70
J	Red	2245	3.31	15.0	40
K	Red Fleshed	2338	3.09	14.5	23
L	Reinking	3805	3.70	15.0	71
M	Siamese	2421	6.04	35.0	7
N	Siamese Acidless	2240	5.94	25.0	7
O	Sweet	3067	3.40	17.5	20
P	Tahitian	3806	3.77	14.0	13
Average				17.9	28.7

[a] CRC # = Citrus Research Center acquisition number.
[b] Unit: ppm.
[c] TG = Total Limonoid Glucosides (ppm).

Table 2—Limonoid aglycones in pummelo seeds[a]

Group[b]	Limonin	Nomilin	Obacunone	Total
A	4,400	2,060	1,050	7,510
C	3,860	812	571	5,243
D	1,670	1,020	370	3,060
G	1,090	281	162	1,533
H	602	111	60	773
I	6,260	2,760	880	9,900
J	1,730	656	361	2,747
K	3,200	1,130	420	4,750
L	551	402	140	1,093
M	1,190	510	201	1,901
O	4,360	3,560	1,450	9,370
P	927	298	251	1,476
Average	2,487	1,133	493	4,113

[a] Unit: ppm on a dry weight basis.
[b] Group B, E, F, N: Not determined.

Table 3—Limonoid glucosides in pummelo seeds[a]

Group[b]	NG[c]	NAG	OG	Total
A	935	632	345	1,912
C	171	191	148	510
D	308	174	102	584
E	310	172	93	575
G	82	45	49	176
H	85	0	45	130
I	616	386	181	1,183
J	546	214	181	941
K	1,264	330	295	1,889
L	127	56	60	243
M	515	100	31	646
N	469	116	34	619
O	428	152	110	690
P	57	91	41	189
Average	422	190	123	735

[a] Unit: μg/g of seed.
[b] Group B, F: Not determined.
[c] G: glucoside, N: nomilin, NA: nomilinic acid, O: obacunone.

juice has a severe limonin bitterness problem. Only one cultivar, Nakon Chaisi, had a concentration of 4 ppm which is below the limonin bitterness taste threshold of 6 ppm (Guadagni et al., 1973). All samples except one became very bitter several hours after juicing, and several became extremely bitter. Two samples exceeded 30 ppm of limonin. No relationship was found between limonin content and physical appearance of fruit.

In addition to the limonin delayed bitterness, pummelo juices also contained considerable levels of bitter flavanone neohesperidosides, mainly naringin (Albach and Redman, 1969; Rouseff, 1987). Some cultivars may include neoeriocitrin, neohesperidin, and poncirin (Nishiura et al., 1971; Kanes et al., 1993). When we tasted the juices immediately after juicing, one juice sample, Pink CRC 2244, was very bitter. This bitterness was undoubtedly due to the presence of a high concentration of bitter flavonoids.

Total limonin glucoside concentrations in pummelo juice samples ranged from 7 to 71 ppm with an average of 29 ppm. These concentrations were much lower than those of grapefruit, lemon, mandarin and orange juices (Fong et al., 1990). For comparison, commercial orange juices contain an average of 320 ppm limonoid glucosides.

Biosynthesis of LARL in citrus fruit tissues occurs until fruit is harvested (Hasegawa et al., 1991; Fong et al. 1992). The accumulation of LARL, however, slows and then decreases during late stages of fruit growth when it converted to nonbitter limonin 17-β-D-glucopyranoside. The concentration of LARL then continues to decrease until fruit is harvested. This conversion, a natural limonoid debittering process, is catalyzed by the enzyme, limonoid UDP-D-glucose transferase. The high concentration of limonin and very low concentration of limonoid glucosides strongly suggest that pummelo fruit has low activity levels of UDP-D-glucose transferase. However, Valencia orange has a very efficient debittering system. Mature Valencia orange accumulates high concentrations of limonoid glucosides and has very low concentrations of aglycones. Valencia orange juice does not present a limonin bitterness problem. Even in navel orange, known for limonoid bitterness in early to mid-season fruit, the presence of the natural debittering system decreases the levels of aglycones and results in lessening of the bitterness in late-season fruit (Hasegawa et al., 1991).

The total limonoid concentrations of pummelo juices averaged 47 ppm, much lower than that of most citrus juices. This indicated that the total activity of limonoid biosynthesis in pummelo fruit was very low in comparison to other citrus. Because of the low UDP-D-glucose transferase activity, pummelo juices and processed products present severe limonoid bitterness problems.

The pH of pummelo juices we examined were generally between 3 and 4 with exception of two cultivars, Siamese and Siamese Acidless. These two had pH ≈6, but their limonin concentrations and total limonoid glucoside concentrations were within the ranges of other cultivars.

Pummelo seeds were also analyzed for limonoid content (Table 2 and 3). TLC analysis showed that seeds contained limonin, nomilin, obacunone, and trace amounts of deacetylnomilin (Table 2), which agreed with results of Nakatani et al. (1989). Two research groups (Hashinaga and Itoo, 1983; Morishita et al. 1985) have reported the presence of deoxylimonin in pummelo seeds, but we did not detected it. Limonin was the major limonoid aglycone in all pummelo seeds analyzed, as in other citrus seeds (Ozaki et al. 1991). Total limonoid aglycone concentrations in seeds ranged from 773 ppm to 9,900 ppm, (average 4,113 ppm, Table 1). Total limonoid glucoside concentrations in the seeds ranged from 130 ppm to 1,912 ppm (average 735 ppm, Table 3). Compared to other citrus seeds, pummelo seeds had very low concentrations of total limonoid glucosides. Nomilin glucoside was the major glucoside in most cultivars. The 10-fold difference in concentrations reflected the variations among cultivars.

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Ms received 3/13/95; revised 5/30/95; accepted 6/17/95.

Ozone Storage Effects on Anthocyanin Content and Fungal Growth in Blackberries

M. MARGARET BARTH, CEN ZHOU, JULIEN MERCIER, and FREDERICK A. PAYNE

ABSTRACT

Ozone exposure was assessed for storage of thornless blackberries which are prone to fungal decay. Blackberries were harvested and stored for 12 days at 2°C in 0.0, 0.1, and 0.3 ppm ozone. Berries were evaluated for fungal decay, anthocyanins, color and peroxidase (POD) activity. Ozone storage suppressed fungal development for 12 days, while 20% of control fruits showed decay. The main mold was *Botrytis cinerea*. Ozone storage did not cause observable injury or defects. By 12 days, anthocyanin content of juice was similar to initial levels for all treatments. Surface color was better retained in 0.1 and 0.3 ppm-stored berries by 5 days and in 0.3 ppm berries by 12 days, by hue angle values. POD was greater in controls and 0.1 ppm samples, and was lowest in 0.3 ppm fruits by 12 days. Ozone storage resulted in market quality extension.

Key Words: ozone, blackberries, anthocyanin, peroxidase, storage

INTRODUCTION

SMALL FRUIT including blackberries have a short market life. Once they are harvested, the time they can be stored at low temperatures is limited by fungal spoilage by *Botrytis cinerea* followed by rapid loss of market quality (Dennis and Mountford, 1975; Dennis, 1983; Sommer, 1985; Ellis et al., 1991). Treatments to maximize shelflife and retard fungal development have been extensively studied (Dennis and Mountford, 1975; El-Kazzaz et al., 1983; Morris et al., 1985; Li and Kader, 1989).

Production and marketing of thornless blackberries has increased. However, blackberries are very susceptible to decay caused by fungi, mainly gray mold rot caused by *B. cinerea* and soft rot caused by *Rhizopus* and *Mucor* spp. (Dennis, 1983; Ellis et al., 1991). Preharvest fungicides have been effective in reducing postharvest fungal infection in small fruit; however, fungicides are under active review in many countries due to their possible health risks. Efforts are being made to replace synthetic fungicides by alternative compounds to control decay and improve shelflife. In addition, strains of *B. cinerea* with resistance to such fungicides have been reported (Maas et al., 1991). As the number of approved fungicides is reduced, there is increased need for technologies which can safely and effectively inhibit fungal growth, reduce postharvest losses, and extend market quality in small fruit.

Ozone (O_3), a strong oxidant, can oxidize contaminants in air and has been demonstrated to limit growth of fungi and abate ethylene in cold rooms (Berger and Hansen, 1965; Heagle, 1973; Dickson et al., 1992). Ozone has been used to control postharvest rot with varying degrees of success in cranberries, strawberries, citrus, peaches and pears (Berger and Hansen, 1965; Norton et al., 1968; Ridley and Sims, 1967; Harding, 1968; Spotts and Cervantes, 1992). Ozone was used as a brief, prestorage or storage treatment in air or water, or as a continuous atmosphere throughout storage. Early reports of ozone storage as a postharvest treatment were conducted before efficient ozone generators were available and before reliable means were available to maintain, measure and control ozone concentrations in coldrooms. Assessment of quality attributes other than fungal growth were often not included and the effects of continuous ozone exposure on fungal growth and quality in small fruits require further investigation. Our objective was to assess continuous ozone exposure (0.0, 0.1, 0.3 ppm) for extending the market life of blackberry fruit.

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MATERIALS & METHODS

Fruit and storage

Blackberry fruit (cv Chester) was produced in field plots at the Univ. of Kentucky. Fruit was harvested, transported to the Univ. of Kentucky Agricultural Engineering Pilot Plant, handled according to commercial practice, weighed into perforated clam shell containers (140g) and placed in three coldrooms controlled at 2°C, 90% RH, and ozone concentrations of 0.0, 0.1, 0.3 ppm. Temperature, relative humidity and ozone concentration of each coldroom was computer-controlled (Fig. 1). The temperature was controlled by the refrigeration system ±1.2°C. A sensor (Rotronic Hygrometer, Series 1H4, Rotronic Instrument Corp., Huntington, NY) was used to measure relative humidity and was checked using a chart hygrometer (The Dickson Co., Addison, IL No. 71297-01). Coldroom temperatures were confirmed with thermometers and by the temperature measurement from the hygrometer. Ozone was produced using ozone generators (Model PA600, Zontec Inc., Ogdensburg, NY). Ozone concentration was measured using a DASIBI UV detector (Model 1003-AH, DASIBI Environmental Corp., Glendale, CA) which had been factory calibrated. The relative humidity and ozone concentration in each coldroom were measured and controlled using OPTOMOX, analog and digital data acquisition board (OPTO 22, Temecula, CA). A computer program using Professional Basic (Microsoft Corporation, Redmond, WA) was written to record the analog data and engage the ozone generator, electric heater, and humidifier. Accuracies were ±5% for relative humidity, and ±0.01 ppm for ozone concentration.

At each sampling interval (0, 1, 2, 5, and 12 days) duplicate containers were removed for analysis of fungal growth, total anthocyanin content, color, and moisture. POD activity was measured at 0, 2, 5, and 12 days storage. Each experiment was repeated 2 times and an analysis of variance was conducted on the data (SAS Institute, Inc., 1985).

Estimation of fungal decay

Fungal infection was estimated visually during the course of each experiment. Blackberry fruit showing surface mycelial development

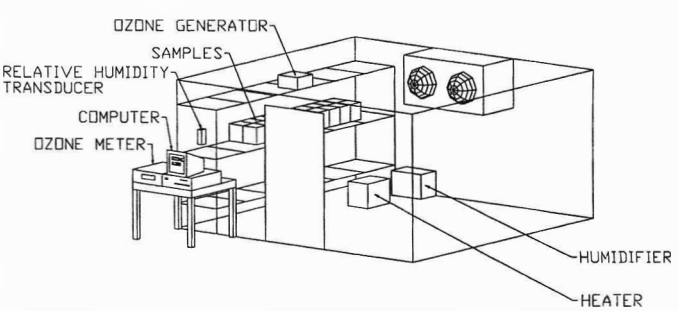

Fig. 1—Refrigeration system used to test the effects of ozone on storage life of blackberries. The humidifier, electric heater and ozone generator were placed inside coldroom and engaged using the computer.

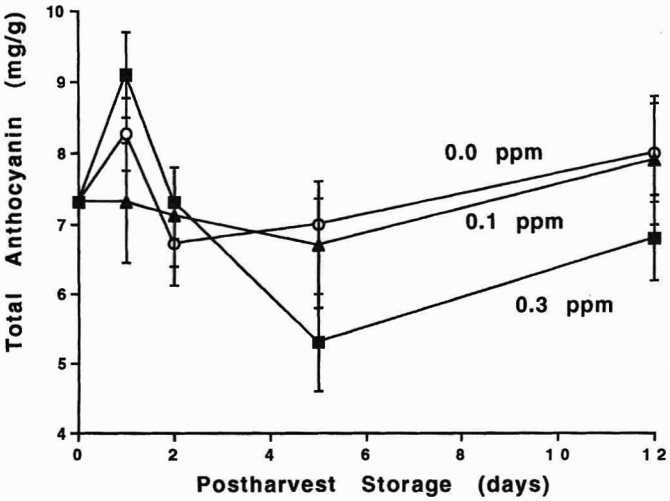

Fig. 2—Ozone effect on total anthocyanin content in blackberry juice over 12 days at 2°C (dry wt). (means ± se, n = 6).

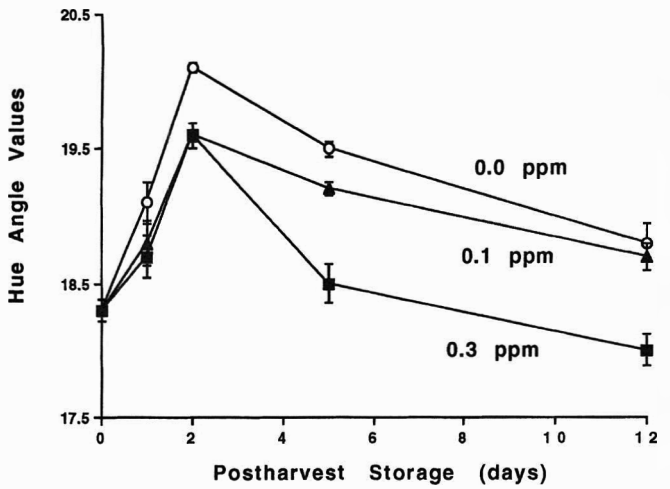

Fig. 3—Ozone effect on Hunter hue angle values in whole blackberries over 12 days at 2°C. (means ± se, n = 6).

were considered decayed. Fungal infection was expressed as percent of berries infected per container. Fungal pathogens were isolated by plating infected berries on potato dextrose agar supplemented with 100 ppm chloramphenicol and identified by visual characteristics (Barnett and Hunter, 1972).

Total anthocyanin content

Total anthocyanin content was determined by spectrophotometric assay (Deubert, 1978). Samples (50g) were placed in stomacher bags and homogenized for 2 min in a Tekmar Lab Stomacher Blender Model 80 (Tekmar, Cincinnati, OH). Aliquots (5 mL) were blended with equal amounts of extracting solution (95% ethanol:1.5M HCl = 85:15) for 2 min at 70 rpm using a Tekmar Tissumizer (Tekmar, Cincinnati, OH). Blackberry extracts (5 mL) were combined with 25 mL extracting solution and filtered using Whatman #54 filter paper. Filtrate was diluted (1:8) with extracting solution and aliquots were taken for spectrophotometric readings at 528 nm and 700 nm. Total anthocyanin content was expressed as mg/g (dry weight).

Color retention

The color of intact, whole blackberry samples was evaluated using a Hunterlab Color/Difference Meter (Model D25-2, Hunterlab, Fairfax, VA) at each sampling interval. The instrument was calibrated with a standard tile (#C2-13717) using an incandescent lamp (D-65,Hunterlab, Fairfax, VA). Each blackberry was placed on the staging platform of the instrument and L, a, b values were measured. Hue angle and chroma values were calculated to assess color changes.

POD activity

POD activity (Hemeda and Klein, 1990) was determined by spectrophotometric assay for homogenized blackberry tissue over storage time. Duplicate homogenized blackberry samples (1g) were placed in 1.5 mL microfuge tubes and blended with 1 mL of 0.1M phosphate buffer (pH 6.8) using a microhomogenizer for 15 sec. Microfuge tubes containing blended samples were placed in a microcentrifuge and spun at 12,000 rpm for 15 min under cold conditions (5°C). Aliquots of supernatant (10 µL) were placed in 1.5 mL reaction cuvettes (1 cm light path) containing 50 µL substrate and 950 µL buffer, inverted once and placed in a spectrophotometer (Model UV160U, Shimadzu Corp., Japan) for absorbance readings for 1 min. Enzyme activity was expressed as change in A_{470}/min/mL fruit extract.

Moisture content

Moisture content was determined on 5g homogenized blackberry samples using a vacuum oven method (AOAC, 1992). Moisture content and percent solids were calculated.

RESULTS

Estimation of fungal decay

Fungal decay was not observed in the ozone-treated fruit by 12 days. In contrast, 20% of controls (0.0 ppm) showed visible signs of fungal growth and decay by day 12. *B. cinerea* was the main cause of infection, although Rhizopus sp. caused some decay in the scar area of the fruits.

Total anthocyanin content

Initial mean total anthocyanin content of juice extracted from whole blackberries was 7.2 mg/g (Fig. 2). Anthocyanin content in blackberries stored in air (0.0 ppm) and at 0.1 ppm remained stable throughout 12 days at 2°C. Anthocyanin content was greater in the 0.3 ppm-treated samples by 1 day (9.1 mg/g); however, a steady decrease was observed by 5 day storage followed by a slight increase by 12 days. No significant differences in total anthocyanin content were observed among treatments over 12 days. By 12 days, total anthocyanin levels in all treatment samples were similar to initial levels.

Color retention

Initial mean Hunter hue angle value of the blackberry samples was 18.3 (Fig. 3). Hue angle values increased for all treatments by 2 days, followed by a gradual decrease by 12 days. Red color of intact, whole blackberry fruit was best retained in 0.3 ppm-treated fruit at 5 and 12 days storage as indicated by hue angle. By 5 days and 12 days, hue angle values in the 0.3 ppm samples were similar to initial values. Although color retention was better in the 0.1 ppm-treated vs control fruit by 5 days, no difference was observed in color retention by 12 days storage. No significant differences in 'L' or chroma values were observed (data not shown).

POD activity

POD activity in blackberry samples over 12 days storage (Fig. 4) showed initial mean activity was 0.13 but declined in all samples during the first 2 days. POD activity in blackberries stored in air (0.0 ppm) dropped sharply during the initial 2 days in storage to 0.05, but increased to ~0.08 by 5 days and remained at that level throughout storage. In blackberries stored at 0.3 ppm ozone, POD activity declined progressively throughout the course of storage remaining significantly lower than the control (0.06). In contrast, exposure to ozone at 0.1 ppm by 2 days caused POD activity to remain higher than the control by 12 days storage and increased to 0.11.

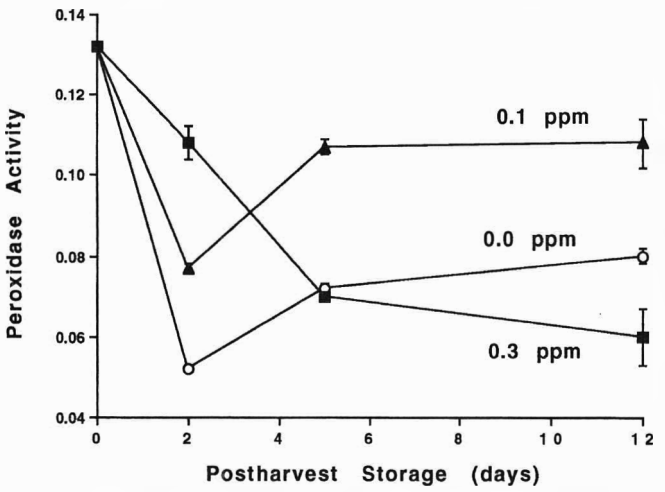

Fig. 4—Ozone effect on peroxidase activity (ΔA_{470}/mL/min) in blackberries over 12 days at 2°C. (means ± se, n = 6).

DISCUSSION

STORAGE OF BLACKBERRY FRUIT under continuous ozone of 0.1 or 0.3 ppm was very effective in preventing fungal decay for up to 12 days at 2°C. Ozonated water was previously shown to inactivate spores of *B. cinerea* (Ogawa et al., 1990; Spotts and Cervantes, 1992). However, short exposure to ozone was not effective in killing spores of *B. cinerea* and other fungi at the wounded surface of tomato and pear fruits (Ogawa et al., 1990; Spotts and Cervantes, 1992). Likewise, a short prestorage exposure to ozone was not effective in preventing decay of pear fruit (Spotts and Cervantes, 1992). It thus appears that constant exposure to ozone throughout storage is more effective in inhibiting storage pathogens as shown here (0.1 and 0.3 ppm, 2°C, 90% RH), and by Harding (1968) for lemons and oranges at 1.0 ppm (14°C, 85% RH), and by Ridley and Sims (1967) in peaches at 0.25 ppm (4–15°C).

Continuous ozone storage at 0.1 and 0.3 ppm significantly extended the market life of thornless blackberries with no observable injuries or decrease in quality over 12 days. While anthocyanin content in fruit stored at 0.1 ppm ozone remained similar to the control, storage at 0.3 ppm resulted in anthocyanin levels fluctuating over time (Fig. 2). An increase in anthocyanin content was observed soon after placement of samples in 0.3 ppm storage. Anthocyanin accumulation was shown previously as a response of plants to ozone (Nouchi and Odaira, 1981). Ozone treatment, especially at 0.3 ppm, had a favorable effect on fruit color, the blackberries remaining significantly redder for most of the storage period (Fig. 3).

POD is implicated in decoloration of plant products. POD has been hypothesized to promote oxidative deterioration of anthocyanins by direct and indirect mechanisms (Grommeck and Markakis, 1964). Increased POD activity in plants has been associated with plants injured by fungi (Kawashima and Uritani, 1963; Johnson and Cunningham, 1972) and ozone (Curtis et al., 1976; Petolino et al., 1983; Patton and Garraway, 1986). POD activity declined in all treatment groups over 12 days storage, but was lower in the 0.3 ppm-treated samples only after 12 days. Changes in POD activity did not appear to correlate with loss of fruit quality. Although increases in POD activity and visible signs of injury are common in various ozone-stressed plant tissues (Patton and Garraway, 1986; Petolino et al., 1983), very different effects on POD activity were observed in blackberries stored at our ozone levels (Fig. 4). Increased POD activity can be associated with ozone damage in plants and could participate in the degradation of anthocyanin pigments. Thus the fate of POD in fruit stored at higher ozone concentrations could have influenced market and sensory quality (Grommek and Markakis, 1964; Patton and Garraway, 1986). Although the control fruit showed visible signs of fungal growth and decay by day 12, POD was slightly higher than that stored at 0.3 ppm and lower than fruit stored at 0.1 ppm. Possibly the POD activity levels we observed were low enough to not significantly affect fruit quality.

Ozone as a storage treatment is a likely alternate to use of fungicides for control of postharvest rot in small fruit. Our computerized control system for ozone storage allowed for maintenance of more constant levels of ozone throughout storage as compared with earlier studies. Ozone storage was beneficial for quality preservation of blackberries and may be effective for quality preservation of other small fruit.

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Ms received 3/29/95; revised 6/30/95; accepted 7/18/95.

Contribution No. 95-05-016 of the Kentucky Agricultural Experiment Station.
Authors express appreciation to Zontec Inc. (Ogdensburg, NY) for technical assistance.

Blanch Temperature/Time Effects on Rheological Properties of Applesauce

A. M. GODFREY USIAK, M. C. BOURNE, and M. A. RAO

ABSTRACT

Peeled and cored 'Idared' and 'Rome' apples were blanched in water for 20, 40, and 60 min at 35°, 47°, 59°, 71°, and 83°C prior to making into applesauce by a conventional process each month from Nov. through March. USDA Consistometer values decreased as blanch temperature increased from 35° to 59°C and increased again from 71° to 83°C. There was little variation in flow behavior index ("n" values). Yield stress increased as blanch temperature increased from 35° to 59°C and decreased from 71° to 83°C. The consistency index (K) and serum viscosity were almost unchanged by blanching temperature but both decreased with increasing storage time of fresh fruit. Blanching apples at 59° to 71°C before making into applesauce gave substantially thicker sauces than unblanched apples.

Key Words: applesauce, apples, rheology, texture, blanching

INTRODUCTION

TECHNOLOGIES for producing high quality, firm canned fruits and vegetables are needed. A low temperature long time (LTLT) blanch (Lee et al., 1979; VanBuren et al., 1960; Chinnery, 1983; Hoogzand and Doesburg, 1961; Steinbuch, 1976; Bourne, 1989) increases the activity of pectin methylesterase (PME), present in fruit tissue, thereby lowering the degree of esterification of pectin and increasing Ca^{++} cross-linking (VanBuren, 1991). Bourne (1992) reported initial experiments using LTLT blanch for processed apple slices and found the LTLT process increased firmness by 50–400% over fruits processed without LTLT blanch. However, results could not be replicated between cultivars, seasons, and blanch times. The changes that take place within the apple during extended storage may be responsible for such inconsistent results. Cultivar differences are also a main factor in apple firmness, storage quality, and processing quality (Smock and Neubert, 1950; McLellan and Massey, 1984; Ananthes-waran, et al., 1985).

Our objective was to provide a better understanding of factors involved in LTLT processing of applesauce and to more thoroughly characterize changes in rheological properties when apples are given a mild heat treatment before processing into applesauce.

MATERIALS

'IDARED' AND 'ROME' processing grade apples were obtained from a local farm. The apples were washed and sorted by size to eliminate those < 63mm in diameter. The apples were stored in a chamber at 0°C, 98%RH until used. About 35 L lots of apples were used for each batch of sauce. A standard applesauce procedure was followed throughout using the method of LaBelle et al. (1960), up to the point of exit from the finisher. A finisher screen with 3 mm holes, and a paddle speed of 800–1000 rpm was used. No water or sugar was added. After exiting the finisher, the sauce was heated to 95–99°C in a closed steam kettle fitted with a mechanical stirrer, and immediately filled into 450 mL cans, rolled for 3 min and then cooled on a spin cooler. Canned sauce was stored at 20°C until opened for testing.

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After peeling and coring, apples were blanched in water in a covered steam jacketed kettle at designated temperatures for prescribed times. Blanch temperatures were 35°, 47°, 59°, 71°, and 83°C. The blanch times were 20, 40, and 60 min. The order of processing, blanch times and blanch temperatures were randomized for each cultivar and storage time. Each cultivar was processed into applesuce after 0, 1, 2, 3, and 4 mo storage. A control, without blanch, was made each month to simulate commercial practice.

METHODS

Consistency and rheology

Consistency of applesauce was measured by the standard USDA procedure, using Consistometer flow sheet no. 1 at room temperature (≈23°C). Sauce was then diluted by adding 5% water, mixed for 45–60 sec using a paddle mixer and consistency was measured as before. Addition of 5% increments of water was continued until the sauce reached an average 6.5 cm flow after 30 sec. A consistometer reading of 6.5 cm is the criterion between USDA Grade A and Grade B sauce. The percent of total water added to the applesauce was recorded.

Yield stress, consistency index, and flow behavior index were measured in duplicate on a Haake RV2 controlled shear rate viscometer (Haake Inc., Saddle Brook, NJ), at 25°C, using a six bladed vane, in a jacketed cylindrical container. Yield stress (σ_0) was measured at 0.4 rpm as the peak dial reading using the vane method (Dzuy and Boger, 1983; Qiu and Rao, 1988). Consistency index was measured by a ramp increase in shear rate from 11.3 rpm to ≈181 rpm, and the power law consistency index and flow behavior index were calculated using a Macintosh desktop computer with FORTRAN programming.

Serum was separated from the sauce by centrifugation and filtration, and its viscosity measured in a glass capillary tube viscometer at 25°C. Soluble solids of serum, as °Brix, was measured with an Abbe' Refractometer (American Optical Corporation, Scientific Instrument Division, Buffalo, NY 14215).

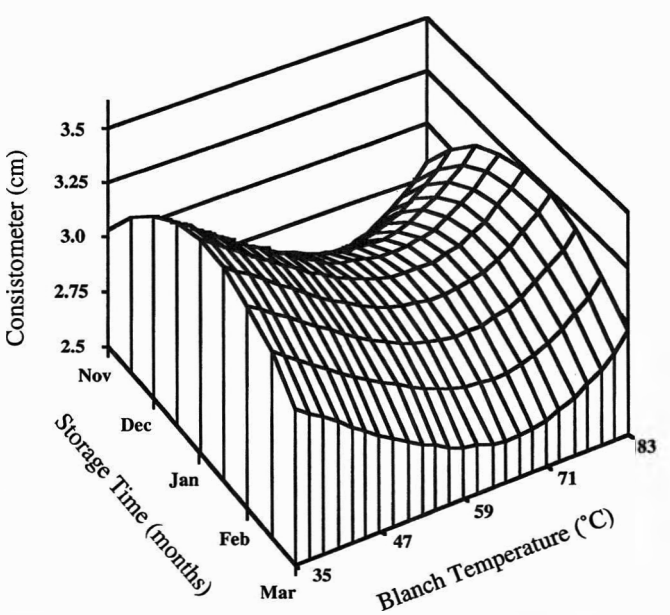

Fig. 1—Prediction model for 'Rome' applesauce consistometer values. Five month raw apple storage period, blanch temperature range 35–83°C, and at any blanch hold times.

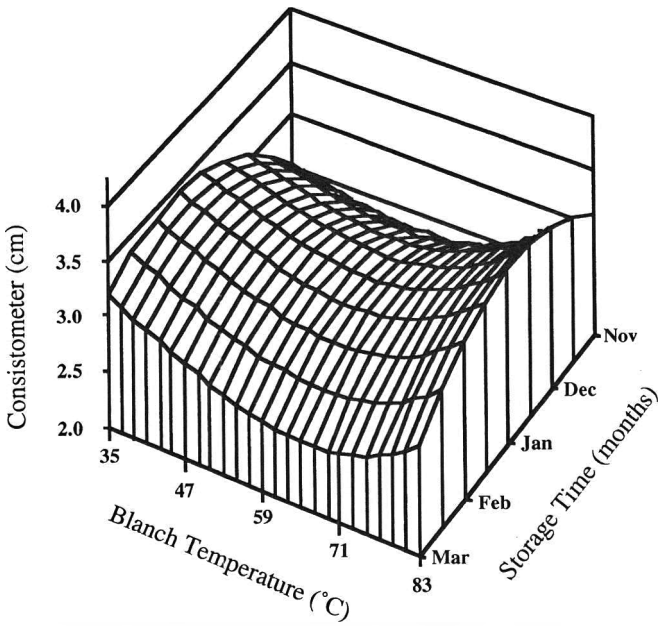

Fig. 2—Prediction model for 'Idared' applesauce consistometer values. Five month raw apple storage period, blanch temperature range 35–83°C, and at any blanch hold time.

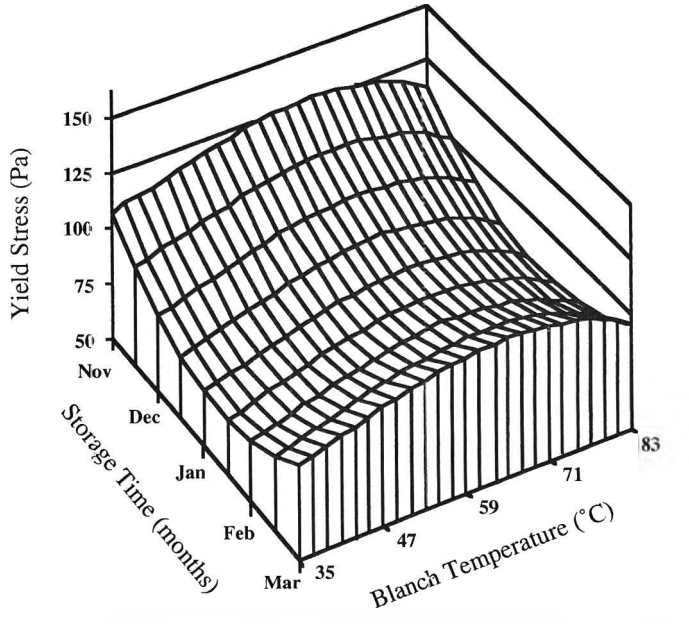

Fig. 4—Prediction model for 'Rome' applesauce yield stress measurements. Five month raw apple storage period, blanch temperature range 35–83°C, and at any blanch hold times.

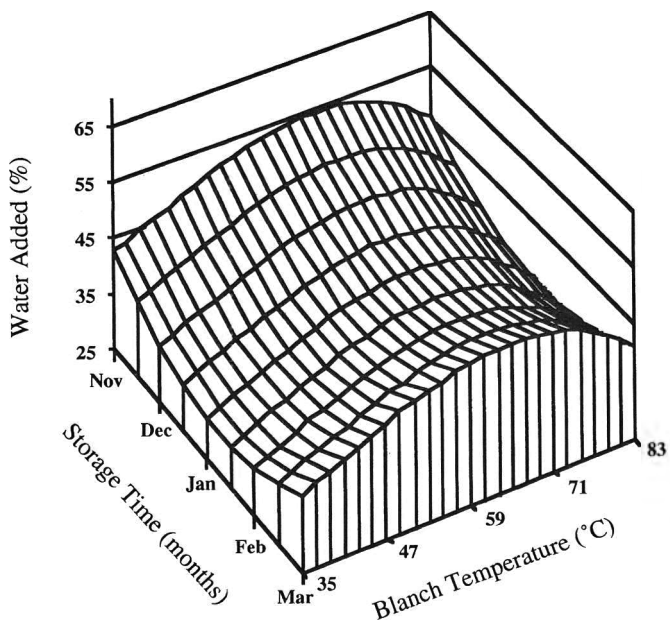

Fig. 3—Prediction model for 'Rome' applesauce percent water added. Five month raw apple storage period, blanch temperature range 35–83°C, and at any blanch hold times.

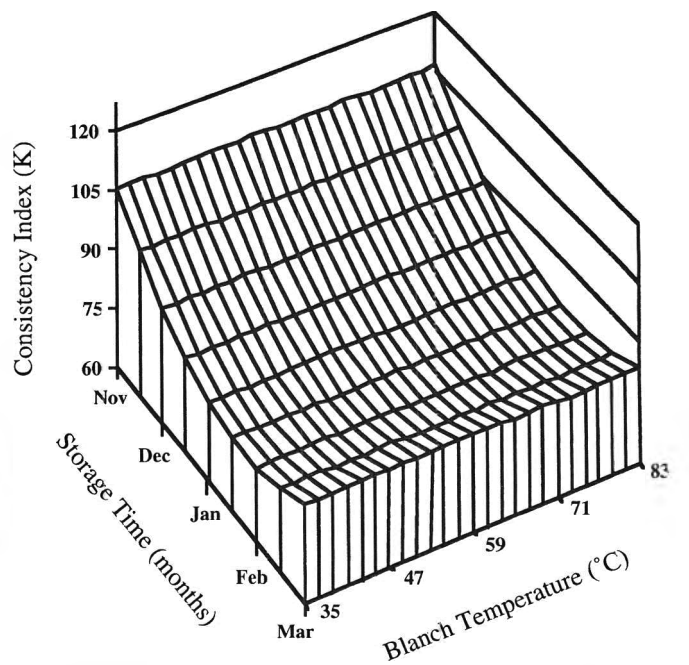

Fig. 5—Prediction model for 'Rome' applesauce consistency index. Five month raw apple storage period, blanch temperature range 35–83°C, and at any blanch hold time.

Statistical procedures

Each cultivar was considered a separate set of data and analyzed using a SAS multifactorial regression model. Independent factors were cultivar, storage time, blanch temperature, and blanch time. Dependent variables were the USDA consistometer readings, average percent water added, consistency index, flow behavior index, yield stress, °Brix, and serum viscosity.

Data were entered into the Prime 9750 computer and were initially subjected to forward and backward stepwise regression, and maximizing R^2 values regression using the SAS System for each of the dependent variables separately. Possible variables included time, temp, month, time*temp, time*month, temp*month, time*time (time2), temp*temp (temp2), month*month (month2), time* time*time (time3), temp*temp*temp (temp3), and month*month*month (month3). The best model for each dependent variable was chosen by maximizing R^2, minimizing the C(p) value, and maintaining significant F-score for each independent variable in the model used. The variables for each model were then entered into a SAS linear regression file to determine the parameter estimates for the prediction model. The parameter estimate prediction line equation was then entered into SAS G3D graphics.

RESULTS & DISCUSSION

SAUCE THICKNESS increased (i.e. Consistometer readings were lower) as temperature increased from 35°C to 59–71°C, and then decreased from 71°C to 83°C. King (1990), and Lee (1969), found apple PME was at its optimum activity around 55–60°C. Lee (1969), also found that PME was inactivated when held at 80°C for 10 min. Our results for Rome (Fig. 1) and Ida Red (Fig. 2) were consistent with the blanch temperature and increased consistency readings they found.

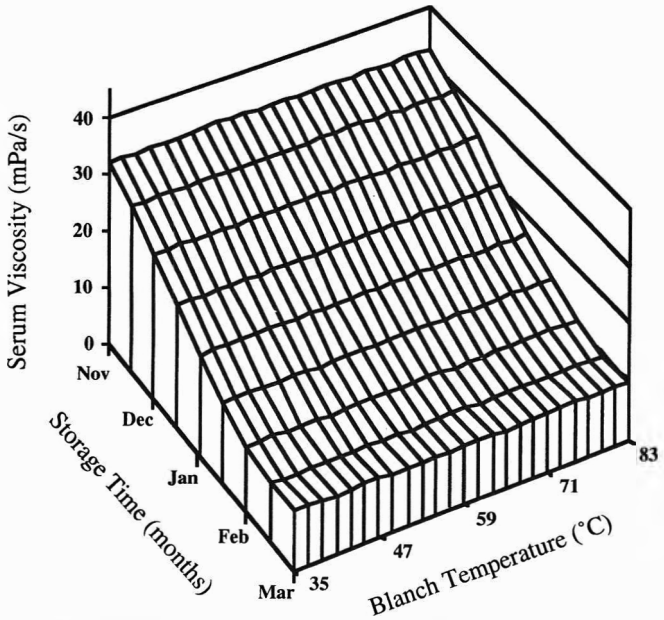

Fig. 6—Prediction model for Rome applesauce serum viscosity. Five month raw apple storage period, blanch temperature range 35–83°C, and at any blanch hold time.

The amount of added water needed to provide a Consistometer reading of 6.5 cm was determined (Fig. 3). It was the inverse of Fig. 1, which was expected because a thick sauce requires more added water to bring it to a standard consistency than does a thin sauce. Changes in yield stress (Fig. 4) gave a curve similar in shape to Fig. 2. The consistency index (Fig. 5) showed little change with temperature.

Thickness of applesauce decreased as storage time progressed from Nov to Jan, and then increased slightly from Jan to March. The Consistency Index (K) decreased from Nov to Feb, and then increased slightly in March. Increases after lengthy storage were unexpected and may have been due to increases in soluble pectin, which is more susceptible to PME activity, due to its high degree of methoxylation. No consistent trends in sauce thickness were found between different blanch times at any given blanch temperature. Some leaching of soluble materials into blanch water occurred. Degrees brix decreased from 10.1 (control) to 7.9 as blanch temperature and blanch time increased to 83°C for 60 min.

Serum viscosity decreased as storage time progressed from Nov to March (Fig. 6), but blanch temperature and blanch time had little effect on serum viscosity. Applesauce serum is primarily sugars and pectins (Rao et al., 1986). Since °Brix and serum viscosity were affected by different variables, changes in serum viscosities could be attributed to pectic changes.

Particle size may also affect consistency measurements between the Haake and Consistometer tests, since the Haake is sensitive to changes in particle size. Firmer apples tend to yield applesauce with larger particle sizes (McLellan and Massey, 1984). Since firmness of the apples changed during storage, particle size of the sauces may have changed, but no measurements of particle size distributions were made. Particle size has been shown to either increase or decrease as storage time progressed for different cultivars (Mohr, 1989).

The flow behavior indexes (n values) were between 0.13 and 0.22, which indicated that the sauces had a high degree of pseudo-plasticity, were highly shear thinning, and far from Newtonian flow where n = 1. Qiu and Rao (1988) found that applesauce with water added to achieve a Bostwick of 4.6 had n values of 0.34–0.37, closer to Newtonian flow than our sauces, in which no water was added. The flow behavior index showed no constant pattern of change with changes in storage time, blanch temperature and blanch time.

The R^2 values that described the prediction models for the dependent variables ranged from 36–79%, and therefore did not account for other factors that were influencing applesauce consistency: particle size, pectin leaching, apple maturity, pectin content, and applesauce processing variations.

CONCLUSIONS

BLANCHING APPLES at 59°C to 71°C for about 20 min before cooking and pulping into applesauce increased sauce thickness considerably. If a constant level of thickness was desired, this blanching treatment increased the yield because more water had to be added to bring the sauce to the standard thickness level.

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Ms received 2/4/95; revised 7/26/95; accepted 7/31/95.

We thank the Comstock Michigan Fruit Division of Curtice-Burns Foods for the gift of the apples used in this experiment, Dr. Karl J. Siebert for guidance with the statistical procedures, and Stanley Comstock for assistance in making the sauces.

Prediction of 5-HMF Formation in an Industrial Apple-Juice Evaporator

STELLA M. TONELLI, ALBERTO F. ERRAZU, JOSÉ A. PORRAS, and JORGE E. LOZANO

ABSTRACT

The rate of formation of 5-hydroxymethylfurfural (5-HMF) in apple juice with soluble solids ranging from 15 to 69°Brix at 100, 104, 108 or 112°C was experimentally determined. Different mechanisms of reaction were proposed and corresponding kinetic models were derived. The model that provided the best fit to experimental data corresponded to a first-order initiation step, followed by an autocatalytic period limited by consumption of reactants. The resulting kinetic equations were incorporated into the mathematical model of a triple-effect apple-juice evaporator. The simulation values obtained for the content of 5-HMF in the concentrated apple juice were in good agreement with observed industrial levels. From simulation runs 5-HMF generation was strongly related to the temperature level at the first evaporation effect.

Key Words: hydroxymethylfurfural, apple juice, kinetics, modeling, evaporation

INTRODUCTION

MULTIPLE-EFFECT EVAPORATORS are used in apple-juice processing plants to reduce water (under vacuum) at relatively low temperatures. However, in industrial practice it is not unusual to have peaks of high temperature at the first evaporation stage, which can lead to undesirable changes in color and flavor of the final product. Such changes are mainly due to the reaction between reducing sugars and amino acids that are naturally present in juices (Babsky et al., 1986). Toribio and Lozano (1987) found that the rate of formation of 5-hydroxy-methyl-2-furfuraldehyde (5-HMF), an intermediate of browning reactions in apple juice, was directly related to the degree of heating. They also reported that the reaction evolution seemed to follow zero-order kinetics, after an induction period where no build-up of 5-HMF was detected. Babsky et al. (1986) found that accumulation of 5-HMF achieved a plateau after long periods of storage.

Process control is applied in the food industry to both improve quality and reduce energy costs (Frost, 1977; Carter and Chen, 1982; Lozano et al., 1984). However, an undesired side effect of classical control schemes is the "overshoot" of temperature, usually induced in the controlled or some intermediate variables during standard instrument adjustments. In order to achieve adequate control, it is very useful to develop an empirical or simulated dynamic model of the process excluding the action of any control loop. Tonelli et al. (1990) developed a computer program which proved useful to simulate the open-loop dynamic response of an industrial triple-effect evaporator for concentration of apple juice.

Our main objectives were: (1) to obtain complementary data on the formation of 5-HMF at high temperatures over extended periods of heating; (2) to consider the mechanism of any undesirable side-reactions and to derive corresponding kinetic equations; (3) to incorporate such equations into the mathematical model of an industrial triple-effect apple-juice evaporator, to provide a reliable steady-state simulation of the unit. The resulting computer program would serve not only to improve evaporator operation, but also to take into account quality aspects of the product.

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MATERIAL & METHODS

THE TYPE OF APPLE JUICE studied and the heating and cooling procedures employed were reported previously (Toribio and Lozano, 1986). The heating apparatus consisted of a set of thin stainless-steel rectangular cells, designed to have a relatively high sample capacity and short come-up times. Fast heating and cooling were obtained by sinking the cells, filled with apple juice, into the appropriate temperature-controlled medium: water-glycerol and water-ethanol for high and low temperatures (up to 112°, and −10°C respectively). Come-up times were less than 40 sec. Apple-juice samples of different concentrations (15, 30, 50, and 70 Brix), were heated at 100, 104, 108, and 112°C, during time intervals from 4 to 120 min. Soluble solids were measured as °Brix in a bench refractometer at 20 ± 0.1°C. The concentration of 5-HMF was determined following the procedure described by the IFFJP (1984), which was based on the colorimetric reaction between barbituric acid, p-toluidine and 5-HMF.

Theoretical considerations

Song et al. (1966) found that, on the basis of kinetic considerations, 5-HMF is a major intermediate in the nonenzymatic browning reaction (NEB) between D-glucose and glycine and proposed the following mechanism to follow melanoidin production.

$$G + g \xrightarrow{k_1} I$$

$$I \xrightarrow{k_2} B_r$$

$$I + b \xrightarrow{k_3} B_m$$

where G = D-glucose; g = glycine; I = intermediates; B_r = colored by-products; B_m = melanoidins; b = glycine; and k_i the corresponding rate constants. However, the formation of 5-HMF was not considered as a main pathway in Maillard reactions. They based this conclusion mainly on the continuous 5-HMF accumulation and the pH of the reaction. Labuza and Baisier (1992) reviewed the published reports on the kinetics of NEB including an extensive discussion of the Maillard reaction and a simplified kinetic-based browning reaction mechanism in foods. They analyzed the reactions from reducing sugars and reactive amines to melanoidins through the Amadori rearrangement, Strecker degradation, formation of fluorescent compounds, etc. They also described the influence of limiting reactants, and summarized the most important kinetic equations describing sugars and amino acids consumption and intermediate and brown products formation. They found that second-order kinetics were rarely used, assuming either the amine or sugar was in excess. They also concluded that it was difficult to compare NEB kinetic data reported from different sources on different amines: sugars ratio, variety and conditions of reactions.

We found that most of the published works on NEB kinetics have been based on the study of pure compounds in solution, which is far from a reasonable model for natural apple juice. In other studies, no induction period was assumed and temperature and pH were not in the same range as those used in actual fruit-juice concentration. Moreover, NEB units have been generally reported as OD or absorbance and not directly related to concentrations of reactant and products. In apple juice concentrate, the ascorbic acid content is very low and production of CO_2 was not observed. Therefore, 5-HMF formation was assumed as the main route of NEB. The mechanisms to be proposed for the 5-HMF formation are based on experimental observations that the rate of accumulation of 5-HMF could generally occurred in three periods (Babsky et al., 1986):

1. A first period, characterized by a markedly slow initiation rate.
2. A well-determined intermediate period, which in terms of the reaction could be described as autocatalytic.

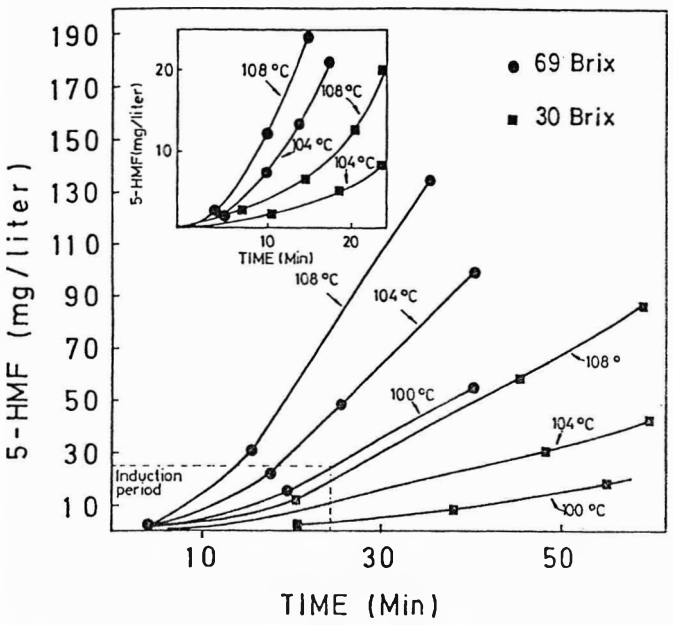

Fig. 1—5-HMF content in apple juice for different solids concentration and temperatures.

3. A final period where production of 5-HMF approaches and reaches a plateau.

We used the following stoichiometric equations which interpret chemical transformations occurring during those three periods.

Initiation step. A number of kinetics models can be in principle proposed for this step, the simplest being:

$$\text{Model I:} \quad A \rightarrow HMF \qquad [1]$$

$$\text{Model II:} \quad A + A \rightarrow HMF \qquad [2]$$

$$\text{Model III:} \quad A + H \rightarrow HMF \qquad [3]$$

$$\text{Model IV:} \quad H + H \rightarrow HMF \qquad [4]$$

$$\text{Model V:} \quad H \rightarrow HMF \qquad [5]$$

where *A, H* and *HMF* represent amino acids, hexoses and 5-HMF, respectively. We could demonstrate that for this particular case Models I and II, which propose reactions involving only molecules of amino acids, are illogical (Reynolds, 1965).

Autocatalytic + final (constant concentration) periods. Transformations occurring during autocatalytic steps are commonly described through mechanistic equations where at least one of the products performs as a reactant. We propose three different models to represent this step (which is characterized by a rapid increase in rate of 5-HMF formation).

However, depending upon the mechanism assumed for development of the observed final constant concentration, two alternatives are possible for each of the three models. Following this reasoning, the following six chemical routes (Eq. 6 to 11) were analyzed:

Case (a) Constant 5-HMF concentration due to consumption of reactives: In this case, the three models which may account for the autocatalytic period were expressed in terms of irreversible reaction steps, i.e.:

$$\text{Model VI:} \quad \begin{cases} HMF + A \rightarrow HMFA^* \\ HMFA^* + H \rightarrow 2\ HMF \end{cases} \qquad [6]$$

$$\text{Model VII:} \quad \begin{cases} HMF + H \rightarrow HMFH^* \\ HMFH^* + A \rightarrow 2\ HMF \end{cases} \qquad [7]$$

$$\text{Model VIII:} \quad \begin{cases} HMF + H \rightarrow HMFH^* \\ HMFH^* + H \rightarrow 2\ HMF \end{cases} \qquad [8]$$

where *HMFA** and *HMFH** are activated complexes. As mentioned, all 3 models (6) to (8) are based on irreversible equations. This means that the plateau only would be reached when the limiting reactant becomes exhausted.

Case (b) Constant 5-HMF concentration due to the nature of the reactions: The final plateau was assumed here to result from equilibrium conditions reached through reversible reactions. Under this assumption, the three possible mechanistic models, similar to the previous ones, are the following:

$$\text{Model IX:} \quad \begin{cases} HMF + A \leftrightarrow HMFA^* \\ HMFA^* + H \leftrightarrow 2\ HMF \end{cases} \qquad [9]$$

$$\text{Model X:} \quad \begin{cases} HMF + H \leftrightarrow HMFH^* \\ HMFH^* + H \leftrightarrow 2\ HMF \end{cases} \qquad [10]$$

$$\text{Model XI:} \quad \begin{cases} HMF + H \leftrightarrow HMFH^* \\ HMFH^* + A \leftrightarrow 2\ HMF \end{cases} \qquad [11]$$

Kinetic expressions which in principle describe the whole process of formation of 5-HMF can be obtained from combinations of any of the different models which represent each of the reaction periods (i.e.: Model III, IV or V combined either with Model VI, VII or VIII, or with Model IX, X or XI).

RESULTS & DISCUSSION

An increase of 5-HMF content occurred in the concentrated apple juice (Fig. 1), in the range 30–69°Brix, at 100, 104, and 108°C (± 0.1°C). To select the best kinetic expression for the rate of 5-HMF formation, values calculated through simulation using these expressions, were compared to the corresponding measured data. For this reason, the cells of the experimental setup were modeled as an isothermal discontinuous constant-volume stirred-tank reactor, i.e.:

$$\frac{d\,C_{HMF}}{dt} = r_{HMF} \qquad [12]$$

where C_{HMF} is the 5-HMF concentration; and r_{HMF} is the reaction rate of formation of 5-HMF. Equation (12) was implemented with each of the different r_{HMF} expressions derived from the 18 possible combinations of the mechanistic models. This led to 18 independent ordinary differential equations. They were integrated under the operation conditions corresponding to each experimental run to obtain the corresponding calculated final 5-HMF concentrations.

Nonlinear regression was performed on the reaction rate parameters for each of the combinations, using the values of 5-HMF concentration measured at the end of the experimental runs. We concluded that the best kinetic model was the one that corresponded to a first-order initiation step (Model V), followed by an autocatalytic period limited by reactants consumption (Model VI):

$$H \xrightarrow{k'_1} HMF \qquad \text{Initiation period}$$

$$HMF + A \xrightarrow{k'_2} HMFA^* \qquad \text{Autocatalytic + final periods}$$

$$HMFA^* + H \xrightarrow{k'_3} 2\ HMF$$

Replacing the selected kinetic model in Eq. (12), the following expression results:

$$\frac{d\,C_{HMF}}{dt} = k'_1\,C_H + k'_2\,C_{HMF}\,C_A \qquad [13]$$

where k'_1, k'_2, k'_3 are the rate constants for step 1, 2 and 3 in reaction mechanism; C_H the hexoses concentration; and C_A the amino acid concentration. This equation was expressed in terms of stable compounds (amino acids, hexoses and 5-HMF), to estimate the evolution of 5-HMF in evaporation stages. Both experimental analysis and mathematical treatment of the experimental data could be further simplified by considering that the concentration of hexoses (C_{HMF}) remained practically constant during heating (Babsky et al., 1986) and proportional to the soluble solids content of the juice:

$$C_H = \beta C_J \qquad [14]$$

Where C_J is the juice concentration and β the initial hexoses

concentration determined from juice concentration. Similarly, the initial amino acids concentration (A_0), can be represented as a function of soluble solids as:

$$C_{A_0} = \alpha C_J \quad [15]$$

where α is the initial amino acids concentration from the juice concentration. The consumption of amino acids could be related to the formation of 5-HMF, as follows:

$$C_A = C_{A_0} - \upsilon' C_{HMF} \quad [16]$$

where υ is a coefficient which accounted both for the stoichiometry of the reaction and for the fraction of amino acids capable of being transformed to 5-HMF. Substitution of Eq. (14–16) in Eq. (13) yielded the final expression:

$$r_{HMF} = k_1 C_J + k_2 (C_J - \upsilon C_{HMF}) C_{HMF} \quad [17]$$

where:

$$k_1 = k'_1 \beta = 1.2348\ 10^{19} \exp(-17373/(T + 273))$$

$$k_2 = k'_2 \alpha = 6.3040\ 10^{6} \exp(-5589/(T + 273)) \quad [18]$$

$$\upsilon = \frac{\upsilon'}{\alpha} = 0.166\ 10^{-7}$$

For the data of Table 2, the kinetic equation (Eq. 17) has the standard deviation, 10.181 and the minimal variance, 101.62. Since kinetics are not a definitive proof of mechanism, two interesting features may be disclosed from the above selection that validate the use of Eq. (17) to represent the rate of formation of 5-HMF during apple juice concentration. The best fit was obtained with a kinetic model which involved a comparatively low number of parameters. In fact, most of the other 17 alternative combinations, particularly those including reversible reactions, led to kinetic expressions far more complex than Eq. (13). From the experimental data we observed that 5-HMF formation in apple juice reached its final plateau simultaneously with the practical disappearance of the amino acids content (Babsky et al., 1986). This undesirable side effect can, in principle, be added to the simulation package of the industrial unit through the following equation:

$$-r_{HMF} V_e^j = F_{ie}^j \frac{1}{\rho_{ie}^j} C_{HMF_{ie}^j} - F_e^j \frac{1}{\rho_e^j} C_{HMF_i^j} \quad [19]$$

Where V_e^j is the evaporation volume at j step, F_{ie}^j and F_e^j are the steam mass flow rate at inlet and outlet respectively, both at j step. The equation accounts for the 5-HMF generation at each evaporation stage. This equation must be solved simultaneously with those corresponding to the steady-state model of the multiple-effect evaporator.

$$F_{ie}^j - F_e^j - F_v^j = 0 \quad [20]$$

$$F_{ie}^j C_{J_{ie}}^j - F_e^j C_{Je}^j = 0 \quad [21]$$

$$F_{ie}^j \Delta H_{ie}^j - F_e^j \Delta H_e^j - F_v^j \Delta H_e^j + Q_e^j = 0 \quad [22]$$

$$F_{ip}^j \Delta H_{ip}^j - F_p^j \Delta H_p^j + Q_p^j = 0 \quad [23]$$

Where F_v^j, F_{ip}^j are the mass flow rates of vapor and preheating inlet; ΔH_{ie}^j, ΔH_e^j, ΔH_p^j is the enthalpy of vapor phase at inlet, outlet and preheating; and Q_e^j, Q_p^j are the heat transfer rate of vapor phase and preheating zone, all at j effect.

The summation of Eq. (19) to (20) to (23) makes it possible to take into account that the industrial evaporation unit, for this particular process, also behaves as a chemical reactor which produces 5-HMF. The computer program we used simulates the operation of a triple-effect Unipektin Flash Concentrator capable of processing about 7600 kg/hr of a 16.3°Brix clarified apple juice. A more detailed description of the simulation model and a flow sheet of the industrial unit were reported earlier (Tonelli et al, 1990).

Table 1—Experimental industrial operating conditions

Operating data	Operating values
Feed flow rate (kg/hr)	7650.0
Feed concentration (kg/kg)	0.16
Feed temperature (°C)	45.0
Steam pressure (kPa)	182.0
First effect steam pressure (kPa)	10.0

Table 2—Simulated formation of 5-HMF as affected by temperature at the first effect

Temperature (°C) (at the 1st effect)	Soluble solids and 5-HMF in the product (at the outlet of the 3rd effect)	
	Soluble solids (kg/kg)	5-HMF 10^3 (kg/kg)
100.1	0.60	5.0
104.4	0.60	7.8
109.1	0.60	12.6
100.1	0.70	6.6
104.4	0.70	10.8
109.2	0.71	16.8
100.0	0.75	7.4
105.1	0.75	12.5
109.1	0.75	18.6

A set of simulation results corresponding to operation conditions detailed in Table 1, are shown in Table 2. Influences of the outlet solids concentration and the first effect temperature on the final 5-HMF generation could be inferred from these results. It can be seen that 5-HMF content in the concentrated juice is a direct function of the degree of evaporation, but most importantly, the results are strongly dependent on the temperature level at the first effect. This conclusion is of great importance because in industrial practice this temperature is not controlled, but usually manipulated to achieve automatic control of the outlet solids concentration.

Also for a reasonable range of industrial conditions (Table 2) the values of 5-HMF predicted from the simulation runs were within the usual industrial measurements reported for this variable (among 5 and 20 mg/L in the concentrated juice). This useful coincidence also validates the kinetic expression developed.

CONCLUSIONS

A KINETIC EXPRESSION for the generation of 5-HMF inside industrial evaporators during the concentration of apple juice was developed. This expression provides acceptable simulation values for the contents of 5-HMF in the product stream. The resulting equation adequately introduced in a steady-state simulation of the industrial unit, provides a useful tool for researchers. Operational and control problems can be analyzed from a standpoint that includes quality aspects of the concentrated juice. The rate expressions we developed should be regarded as empirical correlations, only useful for prediction of rates of reaction within the covered range of experimental conditions.

NOTATION

A	Amino acids
C_A	Amino acids concentration (kg/m³)
C_J	Juice concentration (kg/kg)
C_H	Hexoses concentration (kg/kg)
C_{HMF}	5-hydroxy-methyl-2-furfuraldehyde concentration (kg/m³)
F	Flow rate (kg/hr)
H	Hexoses
5-HMF	5-hydroxy-methyl-2-furfuraldehyde

—Continued on page 1300

L-Ascorbic Acid and Its 2-Phosphorylated Derivatives in Selected Foods: Vitamin C Fortification and Antioxidant Properties

X.Y. WANG, P.A. SEIB, and K.S. RA

ABSTRACT

Two carbonated beverages fortified with 30 mg L-ascorbic acid (AsA) equivalents/250 mL in the form of L-ascorbate 2-monophosphate (AsMP) and stored at 25–35°C retained more vitamin C than those with added AsA or L-ascorbate 2-polyphosphate (AsPP). Breads enriched with ferrous iron and fortified with 560 AsA eq./100g flour in the form of AsPP and AsA retained 40% and 5% vitamin C, respectively, after 6 days at 25°C. Bran (40%) flakes fortified with 0.19% AsA eq. as AsMP and AsPP gave much improved retention of vitamin C during storage for 7 mo at 25–40°C and 7–11% moisture levels. The sodium salts of AsA, AsMP, and AsPP, when injected into whole eye of round beef at 0.18% AsA eq., inhibited hexanal formation after the meat was roasted and stored for 5 days at 5°C. Those sodium salts did not prevent accumulation of peroxides in peanut paste.

Key Words: ascorbic acid, ascorbate phosphate, storage stability, beverages, bread

INTRODUCTION

L-ASCORBIC ACID (AsA) is added to food either to improve quality or to provide vitamin C. AsA has many functions in food, e.g. to stabilize cured meat color, to inhibit enzymic browning, to preserve flavor, and to improve yeast-leavened baked products (Bauernfeind, 1982; 1985).

AsA is distributed widely in plant foods, but it is the least stable vitamin in the food supply. It is also readily leached from food during processing. Crystalline AsA is added to foods (Bauernfeind, 1982) to restore AsA losses, such as those occurring in juices and beverages, potato products, canned fruits, and infant's formula. Addition of AsA as a nutrient in white pan bread has not been successful. More than 50% of AsA was lost in the process of making fortified bread, and almost no AsA survived after storage of bread at 25°C for 7 days (Hung et al, 1987).

Stabilized AsA derivatives have been synthesized (Seib, 1985; Liao and Seib, 1990; Wang and Seib, 1990). L-Ascorbate 2-mono- and 2-polyphosphates (AsMP and AsPP) have high vitamin C activity and high stability towards oxygen (Machlin et al., 1979; Grant et al., 1989). AsMP and AsPP were more stable than AsA during processing and storing of potato flakes, as well as during reconstituting and holding of mashed potatoes (Wang et al., 1990). In food quality applications, AsMP and AsPP were more effective than AsA in inhibiting browning of apple plugs, but not apple juice (Sapers et al., 1989). When used with phosphoric acid, AsMP and AsPP inhibited browning of potato plugs and dice (Sapers et al., 1990). AsMP and AsPP were not effective in inhibiting polyphenol oxidase from mushrooms (Hsu et al., 1988).

Our objective was to examine additional applications of AsMP and AsPP in foods, either as stable forms of vitamin C or as functional ingredients.

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MATERIALS & METHODS

Materials

L-Ascorbic acid (99.6%) was reagent grade from Fisher Scientific Co.; sodium L-ascorbate (NaAs) (99%), t-butylhydroquinone (TBHQ), and propylgallate (PG) were reagent grade from Aldrich Chemical Company, Inc.; and L-ascorbate 2-monophosphate magnesium salt (AsMP) was a gift from Showa Denko K.K. (Tokyo, Japan). Solutions of AsA or its sodium salt were freshly prepared immediately before use. AsMP sodium salt was made from AsMP magnesium salt through cation exchange column chromatography, and was chromatographically pure when examined by high-performance liquid chromatography with uv detection. L-Ascorbate 2-polyphosphate sodium salt (AsPP) was freshly prepared by the method of Liao and Seib (1990). The freshly prepared reaction mixture (pH 10.5) contained 0.77M inorganic sodium phosphates mixed with 0.8M AsA eq. present as the sodium salts of L-ascorbate 2-tri, di-, and monophosphates in a molar ratio of 1/0.04/0.002. Other chemicals were reagent-grade, except where otherwise stated.

Whole eye of round beef (~2 kg each) and Planters™ dry roasted unsalted peanuts were purchased from a local supermarket. Carbonated beverages (Pepsi-Cola™ and Mandarin Orange Slice™) in glass bottles with resealable caps were from a distributor of the Pepsi-Cola Bottling Co., Inc. An instant breakfast cereal, 40% bran flakes, was obtained from a commercial manufacturer. The ingredients in the breakfast cereal listed by the manufacturer were as follows; wheat bran with other parts of wheat, sugar, corn syrup, salt, malt flavoring, butylated hydroxytoluene (BHT, a preservative), as well as vitamins and minerals including iron, vitamin B_3, zinc, vitamin A, vitamin B_6, vitamin B_2, vitamin B_1, folic acid, vitamin B_{12}, and vitamin D.

Enrichment premixes for wheat flour, one containing reduced iron as a fine powder (Type 40, N-Richment-A) and the other ferrous sulfate (Type 540, N-Richment-A), were gifts of the Pennwalt Co. (Buffalo, NY). Those premixes (15.7 mg or 31.6 mg of Type 40 and Type 540, respectively) added to bread flour (100g on a 14% moisture basis) gave enriched flour containing 0.58 or 0.61 mg thiamin, 4.7 or 5.1 mg niacin, 0.4 mg riboflavin, and 3.78 mg iron. Flour of 72% extraction was milled from hard white winter wheat on the pilot flour mill in the Department of Grain Science and Industry. Oriented polyethylene bags were a gift of Printpack Inc. (Grand Prairie, TX).

Determination of free AsA and total AsA

Moisture content was determined by weight loss upon oven drying 1 hr at 130°C. Assay for free AsA was by liquid chromatography under pressure with electrochemical detection as described by Wang et al. (1988). Total AsA was assayed in the same manner after a sample was subjected to phosphatase digestion in the presence of dithiothreitol and compressed yeast (Wang et al., 1988). Levels of phosphorylated AsA (AsMP or AsPP) in food were calculated by difference between total and free AsA. All assays were replicated at least twice.

Vitamin C fortification of carbonated beverages

AsA, AsMP (Mg salt), or AsPP (Na salt) (each 30 mg of AsA eq./250 mL of fluid, or half the adult recommended dietary allowance per serving) was added to a series of resealable bottles of Pepsi™ and Orange Slice™, all bottles were from the same lot. Sodium benzoate (40 ppm) was added to prevent mold growth during storage. The bottles were recapped, and the fortified drinks stored in the dark at 15, 25, and 35°C. Before storage and at regular intervals during 6 wk storage, one bottle of each beverage was assayed for total AsA equivalents.

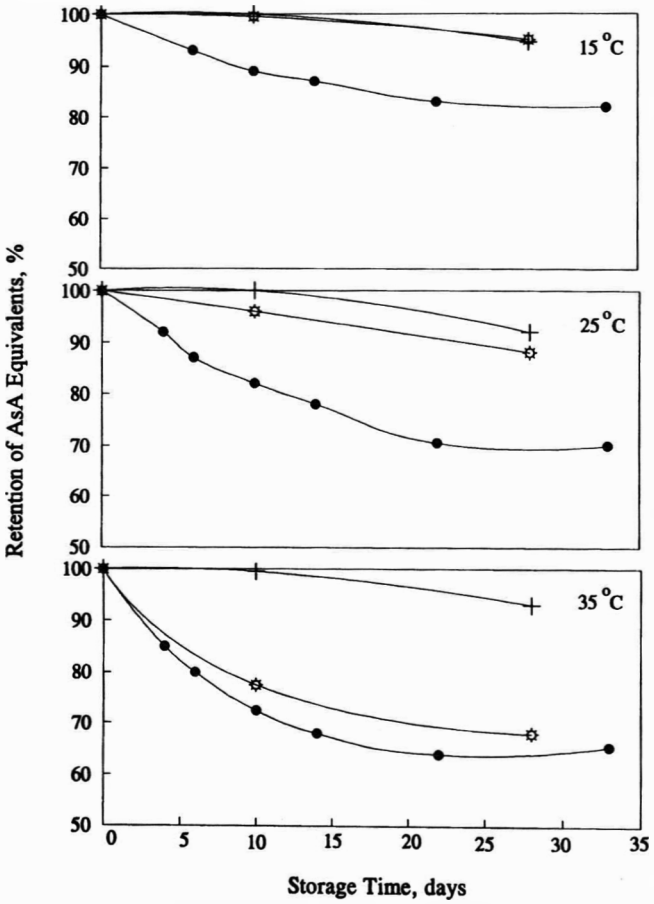

Fig. 1—Stabilities of (•) L-ascorbic acid (AsA), (+) L-ascorbate 2-monophosphate (AsMP), and (★) L-ascorbate 2-polyphosphate (AsPP) in a cola beverage stored at 15, 25, and 35°C. The initial level of vitamin C was 30 mg AsA eq/250 mL.

Vitamin C fortification of white pan bread

Pup-loaves were made according to the procedure of Finney and Barmore (1943). AsA and AsMP (Mg salt) or AsPP (Na salt) and the enrichment premixes containing either reduced iron (15.7 mg Type 40/100 g flour) or ferrous sulfate (31.6 mg Type 540/100g flour) were added at the dough mixing step. AsA was added immediately prior to mixing in solid form, whereas AsMP and AsPP were added as freshly prepared aqueous solutions. Two solutions of both AsMP and AsPP were prepared containing 1.3% and 5.6% AsA eq. Aliquots of 5 and 10 mL of the AsMP solutions and 5 and 7 mL of the AsPP solutions were added at the mixer to give doughs containing 63.8 mg and 560 mg AsA eq. of AsMP/100g flour, or 63.8 and 400 mg AsA eq. of AsPP/100g flour, respectively, on an "as is" moisture basis. Doughs were mixed to optimum, fermented (90% RH) for 180 min at 30°C, moulded, and proofed for 55 min at 30°C. Weights of dough out of the mixer and after proof were recorded. A portion (5-g) of a dough was removed after mixing, fermenting, and final proofing. The dough was assayed for free AsA and total AsA equivalents. After baking for 24 min at 218°C, loaves were cooled for 1 hr at ambient conditions and sealed in polyethylene bags. The loaves were assayed for total AsA eq. before storage or after 3, 5, 8, and 10 days storage at 25°C. A bread slice (~12g) at one-third the distance from the end of a loaf was taken for free and total AsA assays.

Vitamin C fortification of 40% bran flakes

Samples (450g each) of bran flakes with 3.5% moisture content were tumbled and fortified with vitamin C by spraying surfaces with a 12.9, 8.0, or 5.4% sucrose solution containing, respectively, 4.88, 3.02, or 2.04% AsA eq. in the forms of AsA, AsMP, or AsPP, (as sodium salts). The sucrose-vitamin solution was sprayed using an atomizing sprayer (Kontes, 25mL, Fisher Scientific) until the solution add-on amounted to 3.72, 6.01, or 8.89% by weight of the cereal.

After equilibrating in glass jars (2L) at room temperature overnight, each of the three samples of fortified bran flakes was subdivided into 27 small glass jars (145–150g each), which were stored at three temperatures (25, 30, 40°C) for up to 7 mo. Before storage and at regular intervals during storage, samples (~10g each) were removed from each glass jar and assayed for moisture and free or total AsA.

Development of peroxide in peanut butter with added AsA, AsMP, AsPP, *t*-butylhydroquinone (TBHQ), and propylgallate (PG)

Peanut paste was prepared with a Cuisnart food processor (DLC-7M, East Windsop, NJ). Planters® dry roasted unsalted peanuts (200g) were processed with a cutter/mixer blade for 2 min at room temperature tc give a peanut paste with a temperature of 32–35°C. AsA, AsMP (Na salt), AsPP (Na salt), TBHQ, or PG were added during processing at a level of 0.12 mmol/200g, which for the AsA compounds was equal to 0.01% AsA equivalents. The treated peanut paste was weighed (~18g each) into a series of screw-capped glass jars with a head space of ~20 mL. The glass jars were stored in an oven at 63°C for 85 days. Before storage and at different times during storage, a jar was removed and assayed for peroxide value (PV) (AOAC, 1990).

Hexanal formation in refrigerated cooked beef roast containing AsA, AsMP, or AsPP

Whole eye of round beef (~25 × 7 cm) was trimmed to give a cylinder with a length of ~22 cm. The cut beef was injected with 5% (based on meat, "as is" weight basis) deionized water containing either 0.5% sodium tripolyphosphate (STP), 0.18% AsA eq. as sodium AsMP, sodium AsPP, or sodium L-ascorbate (NaAs). The injections were done using a 1 mL hypodermic syringe (B~D Yale Tuberculin syringe, Becton Dickinson & Co., NJ) fitted with a stainless steel needle of lengths 12.5 to 75 mm. Thirty-two radial injections (~0.2 mL each) were made at 12 positions along the length of the meat. The injected beef was stored at 4°C for 16 hr, during which time the meat was massaged several times by hand to promote distribution of injected liquid.

The meat was roasted in a rotary hearth oven at 163°C to an internal temperature of 60°C, which required a cooking time of ≈60 min. After cooking, the roast was trimmed to give even ends and then cut into three equal portions. Before storage and after 3 and 5 days storage at 5°C, a portion was used for total AsA eq. assay and for hexanal assay (Craig et al., 1991).

In a second experiment, whole eye of round roast was injected and cooked using the methods described. The cooked roast was cut into 3 even portions. On day 0, the center portion was sliced into 6 slices each 1 cm thick. Two slices were used for assays of hexanal and total AsA eq. Remaining slices and two portions of roast were stored in a refrigerator at 5°C. After 3 and 5 days, two slices and one portion of roast were used for assays.

RESULTS & DISCUSSION

Stability of Vitamin C in carbonated beverages fortified with AsA, AsMP, and AsPP

AsA was lost somewhat rapidly during the first 5–10 days storage at 15–35°C, but the rate slowed after a 10–20% loss (Fig. 1). Apparently, AsA was destroyed in the cola by both oxidation and dehydration. The initial rapid loss of AsA probably was caused by traces of oxygen in the cola and headspace. The slow loss probably was caused by acid-catalyzed dehydration, which is supported by increased losses at elevated temperatures.

AsMP was more stable in cola than AsA and somewhat more so than AsPP (Fig. 1). About 8% or less of vitamin C was lost in 28 days storage at 15–35°C when AsMP was added as the source of vitamin C compared to 18–36% loss for AsA. AsPP was also more stable than AsA at 15° and 25°C storage. However, at 35°C, AsPP gave about the same loss of vitamin C as AsA. The sample of AsPP we used contained about equimolar levels of AsA and free inorganic phosphate. The inorganic phosphate increases the ionic strength in a beverage, which could accelerate destruction of AsPP.

The carbonated orange beverage contained ≈10 mg AsA/250 mL that had been added by the manufacturer. The vitamin C added at the beginning of storage (30 mg/250 mL) accounted for 75% of the total equivalents of L-ascorbic acid in the beverage (Fig. 2). Losses (5–30%) of vitamin C in the orange bev-

erage fortified with AsA and stored for 4–25 days at 35°C were somewhat below those (10–40%) incurred in the stored cola. However, the losses of AsA in the orange beverage (40%) equalled those in cola after 30 days. Again, AsMP was the most stable form of vitamin C during beverage storage at 25–35°C (Fig. 2).

AsA, AsMP, and AsPP in breadmaking

Wheat flour contains phosphatase, and hydrolysis of the phosphate ester groups on AsMP and AsPP began immediately upon mixing a dough (Table 1). The enzymic hydrolysis of AsPP to free AsA was slower in dough compared to that of AsMP, especially at the high level of addition. After fermentation, when AsMP was added at 560 mg AsA eq./100g flour, 99% of AsMP was hydrolyzed to AsA, whereas 67% AsPP was hydrolyzed (data not shown). After final proofing, when AsPP was added at 400 mg AsA eq./100g flour, the proofed dough contained 17% of its vitamin C level in the form of 2-phosphorylated AsA.

The proportions of L-ascorbate 2-tri- (AsTP), 2-di (AsDP), and 2-monophosphate esters differ in an AsPP preparation depending on its synthesis and isolation. The retention of AsPP in fermented dough would depend on the sample of AsPP. The AsPP in our investigation contained ~95 mole% AsTP.

Retention of vitamin C during storage of bread at 25°C was compared (Fig. 3). When a low level of 63.8 mg of AsA eq. per pup-loaf was added, AsMP and AsPP gave only 3–5% more vitamin C in freshly baked bread than did AsA. Bread made with added AsPP at 63.8 mg eq. AsA retained 5–10% more vitamin C during 6 days storage compared to that with added AsMP and AsA.

When the high levels of 560 mg of AsA and AsMP, and 400 mg AsPP were added per pup-loaf retention of vitamin C immediately after baking increased to above 60%. In addition, the AsA remaining in bread during storage was notably higher when AsMP and AsPP were added to dough rather than AsA. After 6 days storage at 25°C, 32–45% of vitamin C was retained when AsPP and AsMP had been added at 400 and 560 mg AsA equivalents per pup-loaf, respectively, only 17% remained when 560 mg AsA had been added (Fig. 3).

AsMP and AsPP gave more stability in bread during storage than AsA, probably because of a secondary mode of protection. We hypothesize that the orthophosphate ions released from AsMP and AsPP by phosphatase remained in the vicinity of AsA in the dough. Those phosphate ions would chelate metal ions and hinder O_2-oxidation of AsA. Formation of a ternary complex between an L-ascorbate-oxygen-transition metal ion is thought to be the key intermediate leading to O_2-oxidation of AsA (Martell, 1982; Davies, 1992). Chelation of metal ions would inhibit formation of the reaction intermediate. This also could explain why some of the 2-phosphorylated L-ascorbate survived in proofed dough that originally contained 400 mg AsA eq. of AsPP (Table 1); inorganic phosphate is an inhibitor of phosphatase (Tangkongchitr et al., 1982).

When reduced iron was added at the high level of vitamin C fortification, 40% of added AsA was lost during breadmaking (Fig. 4, top) and an additional 40% was lost after 6 days storage of bagged bread at 25°C (Fig. 4, top). Those losses were identical to losses observed in the bread with no added iron. In contrast, when ferrous iron was added for enrichment, 60% of AsA was lost during breadmaking. Almost all AsA was lost after 6 days storage at 25°C (Fig. 4, top).

When high levels of vitamin C were added, ≈40% was lost from fresh bread made with AsMP (Fig. 4). The storage stability of the remaining free AsA in the bread then depended upon the type of iron used. Ferrous iron in the bread caused almost total destruction of the remaining AsA after 6 days storage at 25°C. But reduced iron caused no accelerated destruction and gave about the same (50%) retention of vitamin C after 6 days as that found in the bread made from AsMP with no enrichment (Fig. 4, middle).

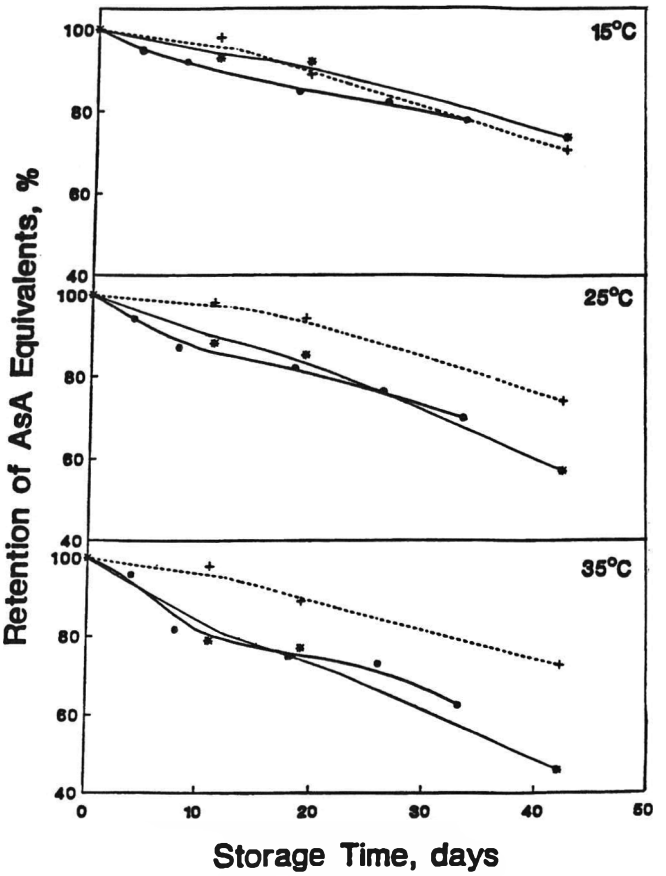

Fig. 2—Stabilities of (•) AsA, (+) AsMP, (*) AsPP in a carbonated orange beverage stored at 15, 25, and 35°C. The beverage contained a mixture of 10 mg free AsA and 30 mg of added phosphorylated AsA eq/250 mL.

Bread made with reduced iron and AsMP at 560 mg AsA eq. per pup-loaf retained 52% vitamin C activity after 6 days storage at 25°C (Fig. 4, middle), compared to 18% vitamin C activity when reduced iron and AsA were added (Fig. 4). Eating one slice (28g) of 4-day-old bread with added AsMP and reduced iron would provide 97% of the adult RDA of vitamin C. One slice of the same bread made with added AsA and reduced iron would provide 37% RDA. Bread fortified with AsMP or AsA at 63.8 mg AsA equivalents per pup-loaf and stored for 5 days at 25°C retained only 13% vitamin C and would provide only 3% RDA of vitamin C in one slice.

Reduced iron did not accelerate destruction of free AsA in bread. However, the absorption of reduced iron in the digestive tract is not promoted by AsA. Previous work has shown that AsA enhances absorption of ionic forms of iron enrichment from foods, including bread (Koch et al., 1987; Morck et al., 1980). The combination of adding an ionic form of iron together with free AsA does not appear feasible in bread, because the transition metal ion catalyzes O_2-oxidation of AsA.

When AsPP was mixed into bread dough at 400 mg eq. of AsA, breadmaking losses of vitamin C were 35% in presence of reduced iron in the dough *vs* 43% in presence of ferrous iron (Fig. 4). Table 1 shows that, after baking, ≈17% of residual vitamin C activity was present in the form of 2-phosphorylated AsA. Storage of bread made with AsPP at 25°C for 6 days resulted in a further 10% decrease in vitamin C level. However, the storage loss was independent of the form of iron enrichment. Apparently, ferrous iron did not accelerate loss of vitamin C in stored bread made with AsPP because of the secondary mode of protecting vitamin C from O_2-oxidation.

Vitamin C fortification of 40% bran flakes

The moisture content of bran flakes was increased from 4% to 7, 9, and 11% by spraying with three vitamin C-sucrose so-

Table 1—Hydrolysis of low and high levels of 2-phosphorylated forms of L-ascorbate during breadmaking[a]

Processing step	64 mg AsA Eq. as AsMP or AsPP added to 100g of flour					
	AsMP			AsPP		
	Free AsA, mg	Total AsA, mg	Vit C retained, %	Free AsA, mg	Total AsA, mg	Vit C retained, %
Before mixing	0	63.8	100	0	63.8	100
After mixing	34.7	51.0	80	13.6	54.3	85
After final proofing	46.4	47.9	75	50.1	51.7	81
After baking	35.1	35.1	55	36.4	36.4	57
	560 mg AsA Eq. as AsMP & 400 mg AsA Eq. as AsPP Added to 100 g of Flour					
	AsMP			AsPP		
Before mixing	0	560	100	0	400	100
After mixing	111	526	94	8	380	95
After final proofing	438	442	79	279	336	84
After baking	348	358	64	195	244	61

[a] Abbreviation: AsA = L-ascorbic acid; AsMP = L-ascorbate 2-monophosphate; AsPP = L-ascorbate-2-polyphosphate.

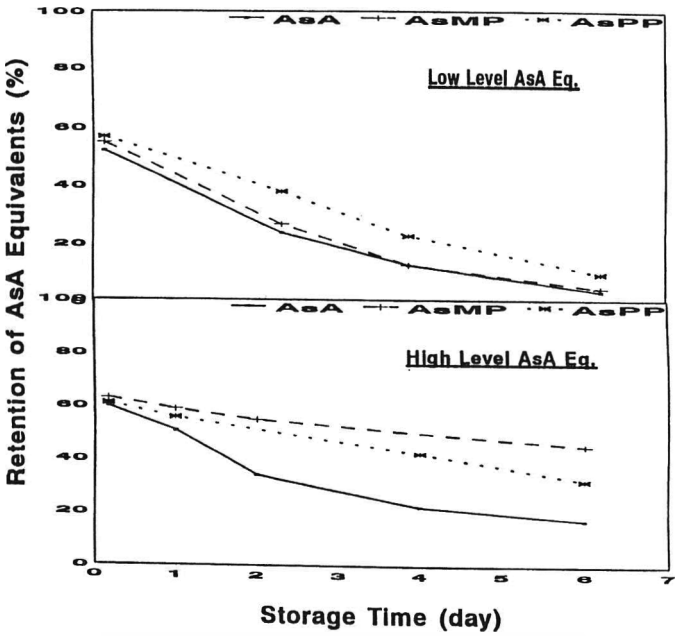

Fig. 3—Retention of vitamin C after bread storage at 25°C. Pup-loaves were baked from a white, pan-bread formula containing 64 (low level) or 560 mg AsA eq. (high level) per 100 g flour in the form of added (■) AsA and (+) AsMP or 400 mg in the form of (×) AsPP.

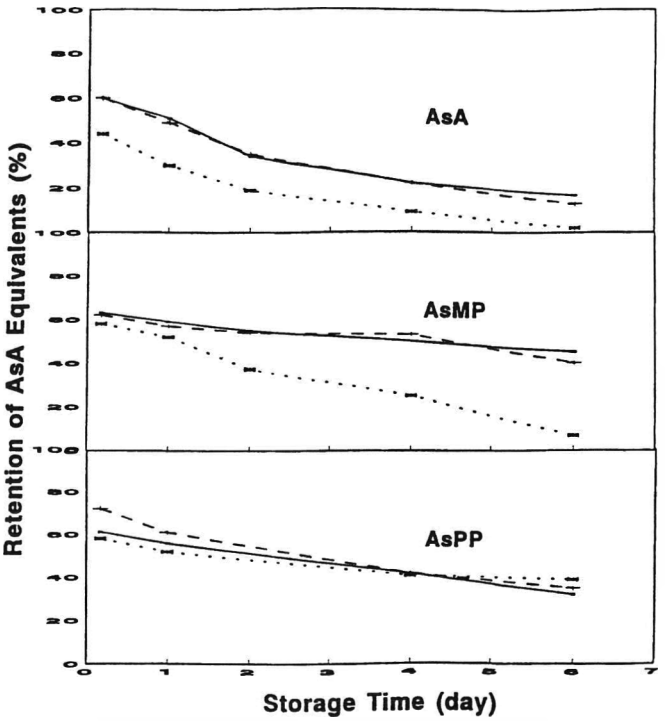

Fig. 4—Retention of vitamin C after storage of bread at 25°C. Pup-loaves were fortified with high levels of vitamin C and contained (*) no iron, (+) reduced iron, and (×) ferrous iron.

lutions, while the levels of vitamin C and sucrose were kept constant, respectively, at 0.19 and 0.50%. Moisture levels above 4% may be desirable in bran flakes to retard hardening of dried fruit mixed with the flakes, while the sucrose coating improves stability of O_2-sensitive vitamins (Borenstein et al., 1990). Of the dry fortified bran flakes in our study 28g would provide 89% of the adult RDA of vitamin C. Moisture levels of the vitamin C-fortified bran flakes did not change during 7 mo storage in glass jars at 25–40°C (data not shown).

Loss of AsA in the bran flakes was rapid (Fig. 5) at high moisture (11%) and temperature (40°C). After 1 mo, about 80% was lost at 11% moisture and 40°C, 75% at 9% moisture and 40°C, 50% at 7% moisture and 40°C, and 5% at 7% moisture and 25°C. Losses were much more rapid during the first 2 mo of storage compared to the last 5 mo.

AsMP was much more stable than AsA under the same storage conditions (Fig. 5 and 6). After 7 mo storage, losses of AsMP ranged from 40% at 9% moisture and 40°C to 0% at 7% moisture and 25–30°C. The data points did not generate smooth curves, indicating that the distribution of AsMP was somewhat nonuniform. When AsPP was added, losses of AsPP after 7 mo ranged from 11% at 7% moisture and 25°C to 60% at 11% moisture and 40°C. Again, the losses increased with increasing moisture and temperature.

Inhibition of oxidation in cooked beef roast during refrigerated storage

The distributions of forms of AsA injected into beef indicted the concentration of AsA eq. differed by ±10% in different regions of the meat (data not given). That injected with AsMP and AsPP also was analyzed for free and 2-phosphorylated AsA. After storage for 16 hr at 4°C, ≈20% of AsMP and AsPP was converted to AsA by phosphatase action.

The hexanal level in the cooked refrigerated meat sample was used as an index of oxidation (meat flavor deterioration) (St. Angelo et al., 1992). When the chunks (~5 cm thick) of roast beef were stored for up to 5 days at 5°C, the hexanal level in the blank sample increased from 0.6 to 2.8 μg/g in Roast A and 0.01 to 1.26 μg/g in Roast B (Table 2). However, the hexanal levels remained low (0.2–0.3 μg/g) in the test samples containing AsMP, AsPP, or NaAsA. In the sliced beef, hexanal increased from 0.01 to 4.77 μg/g after 5 days storage, but it remained low again in samples with added AsMP, AsPP, or NaAsA (0.2 μg/g). In general, the AsA compounds were more effective in reducing hexanal build-up than sodium tripolyphosphate (STP).

Table 2—Cooking loss, moisture, and hexanal in refrigerated beef roast and sliced beef roast[a]

Sample	Treatments[a]				
	Blank	STP	AsMP	AsPP	NaAsA
			Cooking loss (%)		
Roast A	16.3	18.8	13.2	14.2	23.9
Roast B	16.0	16.8	14.9	16.9	18.8
			Moisture (%)		
Roast A	71.5	70.1	70.9	71.5	66.1
Roast B	65.2	66.2	71.1	70.9	68.5
			Hexanal (μg/g) in chunks of beef roast		
Roast A					
No storage	0.60	0.09	0.30	0.08	0.12
3 days at 5°C	0.50	0.30	0.30	0.08	0.22
5 days at 5°C	2.80	0.37	0.30	0.20	0.30
Roast B					
No storage	0.01	0.01	0.01	0.02	0.01
3 days at 5°C	1.42	0.04	0.18	0.02	0.10
5 days at 5°C	1.26	0.04	0.12	0.20	0.30
			Hexanal (μg/g) in slices of beef roast		
No storage	0.01	0.01	0.01	0.02	0.01
3 days at 5°C	3.28	0.40	0.38	0.02	0.12
5 days at 5°C	4.77	0.90	0.20	0.20	0.22

[a] Abbreviations: STP = sodium tripolyphosphate; AsMP, AsPP, and AsA, see Table 1. Test levels were 0.5% STP and 0.18% AsA eq. for AsMP, AsPP, and NaAsA.

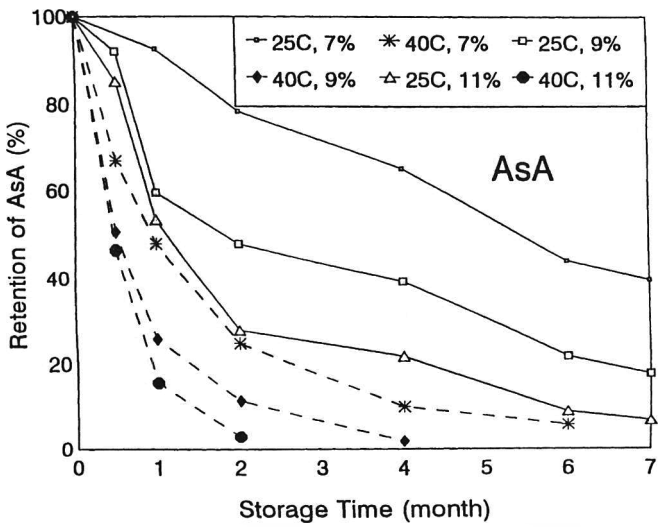

Fig. 5—Retention of vitamin C in 40% bran flakes fortified with 53 mg AsA/28g cereal. The bran flakes contained 7, 9, and 11% of moisture and were stored at 25–40°C.

Table 3—Retention of AsA equivalents after cooking and refrigerated storage of beef roast[a]

Sample	Vit. C source	Retained AsA equivalents in cooked beef roast[b]					
		0 day		3 days		5 days	
		ppm	%	ppm	%	ppm	%
				Chunks of roast			
A	NaAsA	1287	71.5	1564	86.9	958	53.3
B	NaAsA	1538	85.4	879	48.8	1160	64.4
A	AsMP	2391	132.8	1335	74.2	1571	87.3
B	AsMP	1582	87.9	1156	64.2	—	—
A	AsPP	910	50.6	1327	73.7	1399	77.7
B	AsPP	1111	61.7	990	55.0	905	50.3
				Slices of roast			
B	NaAsA	1538	85.4	1021	56.7	1011	56.2
B	AsMP	1582	87.9	1573	87.4	1213	67.4
B	AsPP	1111	61.7	1121	62.3	1171	65.1

[a] Levels of preservatives injected into the raw meat ("as is" moisture basis) were 0.18% AsA (1800 ppm) in the form of the sodium salts of AsA, AsMP, and AsPP. Meat was devoid of AsA prior to injection.
[b] Data are given on "as is" moisture basis.

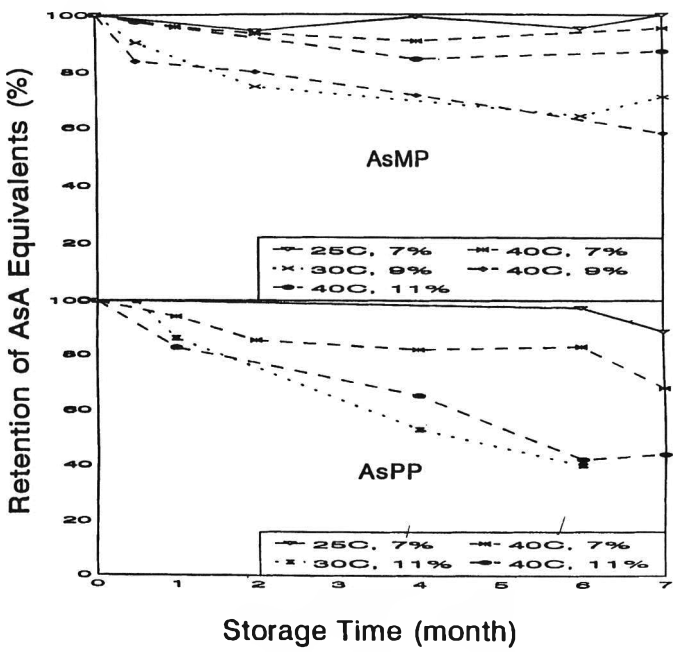

Fig. 6—Retention of vitamin C in 40% bran flakes fortified with 53 mg AsA eq. as AsMP and AsPP and stored at 7-11% moisture content and 25–40°C.

Cooking and storage losses of AsA occurred in the roast beef treated with AsA, AsMP, and AsPP (Table 3). The assay data were somewhat variable because of moisture redistribution and/or instability. Nevertheless, ≈2/3 of AsA eq. were retained after cooking the meat. Losses were probably caused mostly by juice run-off, because losses of AsA from the meat treated with sodium AsA almost equalled those of the meat treated with the stabilized forms of vitamin C (AsMP and AsPP). The high baking temperature, which reduces oxygen solubility in meat, and the metal chelating capacity of protein probably protected sodium L-ascorbate from destruction during roasting. When the meat was treated with AsMP and AsPP, losses of AsA eq. were minor during refrigerated storage after cooking, most likely because of the low temperature.

Sensory data were not taken on the refrigerated cooked beef roast. However, low levels of hexanal in cooked meat indicated that, when beef was injected with solutions of sodium L-ascorbate or 2-phosphorylated L-ascorbate before roasting, off-flavor related to hexanal formation did not develop during 5 days storage at 5°C. ASMP or AsPP compounds provided no advantage over free L-ascorbate to prevent oxidation in the 5-day-old, refrigerated beef roast.

Development of peroxide in peanut butter

Antioxidant can be added to a food at a maximum level of 0.02% by weight of the oil or fat content of the food (CFR 1994). When 0.06 mmol/100g peanut paste of the test compounds was converted to weight percentage, the weight levels were 0.01, 0.02, 0.02, 0.01, and 0.01%, respectively, for AsA, AsMP, AsPP, TBHQ, and PG. After 10 days storage at 63°C, the PV increased rapidly in peanut paste with no antioxidant added (data not shown). TBHQ strongly inhibited fatty acid peroxide formation in the peanut paste up to 50–55 days in the accelerated test, whereas PG and free AsA had only a slight inhibiting effect. AsMP and AsPP had no effect on PV, presumably because of blocking of its 2-OH by a phosphate group, which agreed with results reported by Cort (1974; 1982).

CONCLUSIONS

L-ASCORBATE 2-PHOSPHATE is a stable form of vitamin C because it resists O_2-oxidation. In low- or medium-moisture foods that contain phosphatase, L-ascorbate 2-phosphate gave improved retention of vitamin C. Apparently, nascent orthophosphate inhibited phosphatase and/or chelated pro-oxidant metals.

L-Ascorbate and its 2-phosphorylated ester may prevent oxidation and/or resulting off-flavor in some foods.

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Ms received 2/25/95; revised 6/15/95; accepted 6/24/95.

Contribution no. 95-334-J from the Kansas Agricultural Experiment Station.

We thank John R. Vercellotti and Howard T. Gordon for helpful discussions on peanut paste and fortification of cereal. We also thank P. Ranum of Penwalt for iron enrichments, and Jean Craig and Jane A. Bowers for assistance in experiments on meat.

5-HMF IN APPLE JUICE EVAPORATOR. . .From page 1294

$HMFA^*$	Intermediate between 5-HMF and amino acid
$HMFH^*$	Intermediate between 5-HMF and hexoses
k_1	"Effective" initiation rate constant (kg/(L.- hr.kg/kg))
k_2	"Effective" autocatalytic rate constant 1/(hr.kg/kg)
k_1', k_2', k_3'	Rate constant for step 1, 2, 3 in reaction mechanism
Q	Heat transfer rate (kJ/hr)
r_{HMF}	Reaction rate of formation of 5-HMF (kg/m³)
T	Temperature (°C)
t	Time (hr)
V	Volume (m³)
α	Initial amino acids concentration related from juice concentration, $\frac{C_{AO}}{C_J}$ (kg/m³)
β	Initial hexoses concentration related from juice concentration, $\frac{H_H}{C_J}$
ΔH	Solution enthalpy (kJ/kg)
ρ	Solution density (kg/m³)
υ	Coefficient defined in eqn (17), $\frac{\upsilon'}{\alpha}$ (m³/kg)
υ'	Changes in amino acids concentration due to changes in 5-HMF, $\frac{C_{AO} - C_A}{C_{HMF}}$
Subscripts	
e	Evaporation
i	Inlet
p	Preheating
v	Vapor
Superscripts	
j	Effect number

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Ms received 10/17/94; revised 3/21/95; accepted 4/8/95.

Akara (Fried Cowpea Paste) Quality as Affected by Frying/Reheating Conditions

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ABSTRACT

Three factors (initial cooking time, reheating time, and reheating oil temperature) at three levels each were studied to determine effects on consumer acceptance of partially cooked, frozen, reheated akara. Based on consumer panel results, initial cooking time should be more than 50 sec to obtain acceptable akara. With 75 sec initial cooking time (ICT), reheating time (RT) should be at least 50 sec, while reheating oil temperature (ROT) should range from 152–180°C. RT should be more than 70 sec and ROT not exceed 180°C when 100 sec ICT is applied. Trained sensory panel testing and instrumental/chemical measurements verified that the quality of akara from recommended processing conditions was close to that of fresh akara.

Key Words: akara, cowpea paste, flour, texture, stability

INTRODUCTION

DEEP-FAT FRYING is an important unit operation in the catering and food processing industries. It is important to suppliers of oils, ingredients, fried foods, and process equipment; foodservice outlets; and quality control laboratories (Blumenthal, 1991). Deep-fat frying can produce foods for immediate consumption or for additional processing (Blumenthal and Stier, 1991). Although fried foods have high acceptance by the public, poor preparation of deep-fat fried products is widespread. The common mistakes or mishandling practices which contribute to low quality of fried foods include (1) incorrect temperatures; and (2) use of deteriorated fats (Thorner, 1973). To study the frying performance in a specific unit operation, the widely used 9- or 10-point hedonic scale can be useful, particularly if for assigning numbers to the scale and using such numbers for statistical and regression studies (Jacobson, 1991).

Akara is a fried product from cowpea (*Vigna unguiculata*), which is a primary food legume in West Africa, with more than 90% of the world crop produced in that region. In Nigeria, cowpeas are a principal source of dietary protein and are consumed as boiled, steamed and fried foods (Hung et al., 1990). Varied and sophisticated African cuisine products are based on cowpea paste (Dovlo et al., 1976).

Seasoned with chopped peppers, onion and salt, cowpea paste is whipped, dispensed by spoonful portions into hot oil, and fried into akara balls. Akara is prepared in the home and by street vendors in the marketplace as a breakfast or snack food. Relatively unknown to the American palate, it has been indicated as the most common cowpea-based food product in Africa (Reber et al., 1983). Previous studies show this product can be prepared with equipment readily available in developed countries and has potential for home and institutional use. Consumer acceptance tests conducted with 450 consumers in the metropolitan area of Atlanta, Georgia in 1988 indicated that the product was favorably received by segments of the population (McWatters et al., 1990).

Akara can be used as a freshly-prepared or frozen food or as a bread-like accompaniment for fish and poultry dishes (McWatters and Brantley, 1982). It contains more protein than deep-fat fried cornmeal and potato products (McWatters and Flora, 1980; McWatters et al., 1993). As ethnic and vegetarian foods increase in popularity, akara—which is considered both ethnic and vegetarian—should appeal to potential consumers such as vegetarians and West Africans.

Although akara was perceived to be most marketable as a pre-cooked, frozen, reheatable product such as a frozen supermarket item (McWatters et al., 1992), one of the constraints to its wider use has been that frozen, reheated akara was not comparable to the freshly prepared product. McWatters et al. (1992) indicated that it was important to determine (1) thawing/reheating conditions for pre-cooked, reheatable product for use in institutional food service or home situations, and (2) whether a "fresh-fried" product has more consumer appeal than one which has been pre-cooked and reheated.

Our preliminary study showed the possibility to serve akara as (a) a fully cooked, frozen product, to be reheated by microwave or conventional oven; or (b) a partially cooked, frozen item, to be reheated by finish frying. However, the quality of end products varied with initial cooking and reheating conditions.

Our objectives were to (1) evaluate the effect of initial cooking time, reheating oil temperature, and reheating time on quality attributes of frozen, reheated akara by a consumer acceptance test; and (2) evaluate the quality of akara reheated by different methods (frying, microwaving, and baking) using both instrumental/chemical and sensory methods.

MATERIALS & METHODS

Preparation of cowpea flour and akara

Cowpeas (*Vigna unguiculata*, cv. California blackeye 5) obtained from Pennington Seed Co. (Madison, GA) were held at 2°C until used. Whipped cowpea paste was made from dry cowpea seeds based on the recipe and procedure described by McWatters et al. (1991). The mixture was then dispensed into a continuous fryer (Donut Mini-Matic 110, Belshaw Bros., Inc., Seattle, WA) by using an ice cream scoop (size 40) and fried in peanut oil at 193°C for 50, 75 or 100 sec. Fried products were drained on absorbent paper, cooled to room temperature, packaged in ≈4L Ziploc® freezer bags, sealed and stored at −18°C for 4 wk until subsequent reheating studies.

Consumer evaluation of akara reheated by frying

A 3×3×3 full factorial experimental design representing 3 factors each at 3 levels (initial cooking time at 50, 75, and 100 sec; reheating oil temperature at 149, 166, and 183°C; reheating time at 30, 60, and 90 sec) was used to study the relative contribution of each variable to akara quality. The experimental design, which consisted of 27 treatments, was replicated twice. Reheated akara samples were randomly assigned to panelists with each treatment evaluated by at least 24 consumers (Anon., 1981).

Seventy six consumers consisting of local residents of Griffin, Lovejoy, and Fayetteville, GA, staff and students from the Georgia Agricultural Experiment Station were recruited to evaluate the akara. Panelists were prescreened as regular consumers of fast food and fried products.

Frozen/thawed akara balls were reheated by frying in peanut oil and held under three heat lamps (37.5W, Model 3LOR, Keating Inc., Chicago, IL) for < 10 min before being evaluated by panelists. The test was conducted in eight sessions in two consecutive days. Two sets of five samples each from the 27 treatments were presented to each panelist, with a 10-min compulsory break between sets. In order to prevent sen-

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Table 1—The attributes and intensity of standards used to evaluate quality of akara

Attributes	Standards	Intensity[a]
Color intensity	light yellow card	4.5
	yellow card	10.0
	dark brown card	13.5
Moistness	cracker	0
	apple	7.5
	ham	9.0
Beany flavor	roasted peanuts	1.5
	boiled cowpea seed	6.0
	cowpea meal	13.5
Oiliness	fresh fry	4.0
	peanut butter	9.0
	peanut oil	15.0
Crispness	Tater Tot®	7.5
	onion ring	9.5
Sponginess	dinner roll	5.5
	angel cake	14.0

[a] Intensity ratings based on 15-cm unstructured line scales.

sory fatigue, panelists were advised to take a bite of each sample, taste it, and then expectorate it. They were also instructed to eat a piece of cracker (unsalted saltine crackers, Nabisco Foods, Inc., E. Hanover, NJ) and rinse their mouths between samples.

Panelists were asked to taste akara and rate their degree of like or dislike on a 9-point hedonic scale ranging from 1 (dislike extremely) to 9 (like extremely) by marking the box corresponding to their perceived acceptability rating of an attribute. Those attributes evaluated were color, overall quality, flavor, and texture. Oiliness, from extremely oily (rating = 1) to extremely dry (rating = 9), and willingness to purchase (yes or no) were also assessed for each sample.

Trained panel analysis

Quality of six different frying/reheating treatments (two of which were considered optimum and four considered undesirable based on results from the consumer study), plus microwave reheating, baking reheating, and fresh akara were evaluated by using a trained panel consisting of six males and four females, 18–60 years of age. The panelists were prescreened as regular consumers of fast food and fried products. For baking and microwave reheating, akara was fully cooked by frying in peanut oil at 193°C for 140 sec and then frozen. The microwave reheating condition for 12 akara balls in a covered container with apertures in the lid was 45 sec at 800W power level; reheating by baking was at 204°C for 5 min in a conventional oven. A total of nine treatments was evaluated.

Training was conducted in two sessions of 2 hr each. Six quality attributes (color intensity, moistness, beany flavor, oiliness, crispness and sponginess) were evaluated by using a 15-cm unstructured line scale with standards marked. The standards for each attribute developed by panelists are shown (Table 1). All samples were coded with 3-digit numbers and presented to panelists in monadic order with all standards available upon request for evaluation.

Instrumental/chemical measurements

Color. The lightness (L), redness to greenness (a) and yellowness to blueness (b) values of akara balls were measured by using a Gardner XL-845 colorimeter standardized with a yellow reference tile (L = 78.26, a = −2.04, b = 23.45). Chroma [$C = (a^2 + b^2)^{1/2}$] and hue angle [$\tan^{-1}(b/a)$] were calculated from L, a, b values (Anon., 1979). Each treatment was quadruplicated, and the average was determined.

Moisture. Moisture content was determined as the loss of weight from 5-g samples after drying at 70°C under a vacuum of 74 cm Hg for about 24 hr (McWatters, 1983). Triplicate measurements for each treatment were made for all nine treatments.

Crude fat. Moisture-free samples were used to determine crude fat content by extracting for 24 hr with petroleum ether in a Goldfisch apparatus (McWatters, 1983). Dry basis and wet basis crude fat content were calculated based on weight of dry or wet sample. Each treatment was triplicated, and the average was determined.

Shear force. An Instron universal testing machine (model 1122, Instron Corp., Canton, MA) was used to determine textural measurements. The Instron was fitted with a Kramer shear-compression cell (Hung and Chinnan, 1989) and operated at a crosshead speed of 50 mm/min and a chart speed of 100 mm/min with a 500-kg load cell. One akara ball was placed in the center of the Kramer shear-compression cell for each testing. Peak force required to shear and compress the akara ball was recorded, and each treatment was quadruplicated.

Puncture force. An 11-mm diameter probe was fitted to the Instron to determine the force required to puncture through the crust of individual akara balls. The Instron was operated at a crosshead speed of 50 mm/min and a chart speed of 100 mm/min with a 10-kg load cell. The probe was allowed to penetrate into an akara ball for about 10 mm and the peak force was recorded. Quadruplicated measurements for each treatment were determined for all treatments.

Statistical analysis

Statistical analysis was performed using the Statistical Analysis System (SAS Institute, Inc., 1985). Analysis of variance using the GLM procedure and Duncan's multiple range test was performed to determine the effect of initial cooking time, reheating oil temperature, and reheating time on consumer acceptance. The significance level was established at $p \leq 0.05$.

A second order polynomial model was fitted to the consumer acceptance test data by using Response Surface Regression Analysis (PROC RSREG) to determine the behavior of the response variables in relation to the set of factors (i.e., independent variables). This model can be expressed as follows:

$$E(Y) = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j$$

In this study, k = 3, E(Y) = the expected value of the response variable, X_1 = reheating oil temperature, X_2 = initial cooking time, X_3 = reheating time.

Stepwise Regression Analysis (PROC STEPWISE) (Draper and Smith, 1981) was used to obtain a suitable prediction equation for those response variables significant at the 1% level. Only those independent variables significant at $p < 0.10$ were retained in the prediction model for specific parameters.

The correlation between sensory (trained panel test) data and instrumental/chemical measurements was determined by Regression Analysis (PROC REG).

RESULTS & DISCUSSION

Modeling consumer acceptance

The consumer responses (crust color, overall quality, flavor, texture, oiliness and willingness to purchase) as affected by initial cooking time, reheating oil temperature, and reheating time were compared (Table 2). Results indicated that the crust color of akara reheated at 166°C was generally more acceptable to consumers than akara reheated at 149°C, while the initial cooking time and reheating time had no influence on crust color. The overall quality of akara with 90 s initial cooking time was more favored by consumers than the 30 sec reheating time. Flavor and willingness to purchase ratings were not affected by any of these three factors alone. However, consumers preferred the texture of akara with a longer initial cooking time and reheating time. Akara reheated at 149°C received a lower texture rating compared with akara reheated at 166°C and 183°C. Oiliness was also affected by reheating oil temperature; akara reheated at lower oil temperature was rated more oily (lower oiliness rating) than products reheated at higher oil temperatures, and akara with shorter initial cooking and reheating times was perceived as more oily than that with longer cooking times. Based on overall quality and willingness to purchase as selection criteria, five treatments received favorable responses with an overall quality rating >6.0 and more than 50% of the consumers who evaluated the products indicating a willingness to purchase (Table 3). Three treatments received the least favorable responses with an overall quality rating <5.5 and a willingness to purchase of ≤ 35% (Table 3).

Significance of second order degree polynomial equations fitted to the consumer acceptance responses indicated that only significant regression models for overall quality, texture, and oiliness could be obtained at the $p \leq 0.01$ level. Not all of the variables in the full models contributed significantly to the prediction of attributes. STEPWISE regression analysis of the data

Table 2—Mean values of consumer responses as affected by reheating oil temperature (ROT), initial cooking time (ICT), and reheating time (RT)

Variable	Level	Color[d]	Overall quality[d]	Flavor[d]	Texture[d]	Oiliness[d]	Willingness to purchase[d] (%)
ROT (°C)	149	6.7[b]	5.8[a]	5.9[a]	5.8[b]	2.8[c]	38.0[a]
	166	7.1[a]	6.0[a]	6.0[a]	6.2[a]	3.2[b]	41.9[a]
	183	6.9[ab]	6.1[a]	6.0[a]	6.2[a]	3.7[a]	46.2[a]
ICT (sec)	50	6.8[a]	5.8[b]	5.9[a]	5.9[b]	3.0[b]	38.9[a]
	75	6.9[a]	6.1[a]	6.0[a]	6.1[a]	3.3[a]	43.6[a]
	100	7.0[a]	6.1[a]	6.1[a]	6.2[a]	3.3[a]	43.6[a]
RT (sec)	30	6.8[a]	5.7[b]	5.9[b]	5.9[b]	3.0[b]	39.3[b]
	60	7.0[a]	6.0[a]	6.0[ab]	6.1[ab]	3.2[b]	40.6[ab]
	90	6.9[a]	6.1[a]	6.2[a]	6.3[a]	3.5[a]	46.2[a]

[a,b,c] Fisher's LSD test was employed for mean separation. Values for a specific variable followed by the same letter in a column are not significantly different ($p < 0.05$).
[d] Scales for color, overall quality, flavor and texture were 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely; scale for oiliness was 1 = extremely oily, 5 = just right, 9 = extremely dry; willingness to purchase was calculated as percentage of consumers who would like to buy the product.

Table 3—Overall quality ratings and willingness to purchase responses as affected by selected treatment conditions

End product quality	Treatment	ROT[a] (°C)	ICT[a] (sec)	RT[a] (sec)	Overall quality[b]	Willingness to purchase[c] (%)
Desirable	1	166	75	30	6.5	65
	2	149	75	60	6.5	54
	3	166	75	90	6.1	58
	4	166	100	90	6.5	50
	5	183	100	90	6.2	62
Undesirable	1	149	50	30	4.9	27
	2	149	50	60	5.4	35
	3	149	75	30	5.2	31

[a] ROT = Reheating oil temperature (°C), ICT = Initial cooking time (sec), RT: Reheating time (sec).
[b] Scale for overall quality was 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely.
[c] Willingness to purchase was calculated as percentage of consumers who would like to buy the product.

Table 4—Selected prediction equations for akara quality parameters obtained by STEPWISE regression analysis

Akara quality parameters	Prediction equation[a]	R^2
Overall quality[b]	$-1.418 + 0.03972X_1 + 0.005299X_2 + 0.09015X_3 - 0.0005027X_1X_3$	0.37
Texture[b]	$-22.84 + 0.3009X_1 + 0.006923X_2 + 0.07645X_3 - 0.0007911X_1^2 - 0.0004211X_1X_3$	0.51
Oiliness[b]	$-10.79 + 0.06926X_1 + 0.1107X_2 + 0.05129X_3 - 0.0003180X_2^2 - 0.0003469X_1X_2 - 0.0002640X_1X_3$	0.67

[a] X_1 = Reheating oil temperature (°C), X_2 = Initial cooking time (sec), X_3 = Reheating time (sec).
[b] Significant at $p < 0.10$ level.

resulted in reduced models with important variables at $p \leq 0.10$. Selected prediction equations (Table 4) were developed for overall quality, texture, and oiliness as a function of reheating oil temperature (°C), initial cooking time (sec), and reheating time (sec).

Contour plots of overall quality, texture, and oiliness at 75 sec and 100 sec initial cooking time were developed as a function of reheating oil temperature and reheating time (Fig. 1–2). The best overall quality rating (6.3) of reheated akara could be obtained under the conditions shown in the upper portion of the contour plots (Fig. 1a, 2a). However, for 50 sec initial cooking, the highest overall quality rating that could be obtained was 6.0 (data not shown). Up to a reheating oil temperature of 170°C, texture ratings increased with increasing reheating time (Fig. 1b, 2b). High reheating oil temperature and long reheating time resulted in high oiliness ratings (less oily: Fig. 1c, 2c). Low oiliness was favored by most consumers as indicated by written comments.

Contour plots corresponding to each initial cooking time were superimposed to determine optimum processing conditions (Fig. 3). The constraints for optimization of the product were: overall quality ≥ 6.25, texture ≥ 6.30, and oiliness ≥ 3.25. In our study, products that met all three of these conditions were considered acceptable. We superimposed the previous contour plots of texture, oiliness, and overall quality at the 75 sec initial cooking time (Fig. 3a). This plot suggested that at 75 sec initial cooking, reheating time should be ≥ 50 sec, while the reheating oil temperature should range from about 152°C to 180°C. In general, the lower the reheating oil temperature, the longer the reheating time required. To obtain acceptable akara quality, the reheating oil temperature should not be greater than 180°C (Fig. 3b) and reheating time must be at least 70 sec when the 100 sec initial cooking time is applied. The overlapping contour map for 50 sec initial cooking could not be obtained. That is, there was no processing condition at 50 sec initial cooking time which fit the above constraints. Comparing the overlapped areas in the 75 and 100 sec initial cooking time contour maps, the reheating condition for samples from 75 sec initial cooking was more flexible (larger shaded area) than that of 100 sec initial cooking time.

Overall quality and willingness to purchase also significantly correlated with color, flavor, texture, and oiliness. The strongest linear relationship existed between overall quality and texture ($r = 0.78$) and between willingness to purchase and flavor ($r = 0.51$). However, if overall quality and willingness to purchase were correlated with both texture and flavor, the correlation coefficients were improved to 0.85 and 0.57, respectively. The linear predictive equations were: $Y = 0.39X1 + 0.59X2 + 0.08$ (Y-overall quality, X1-flavor, X2-texture) and $Y = 10X1 + 9X2 - 74$ (Y-willingness to purchase %, X1-flavor, X2-texture). These indicated that overall quality and willingness to purchase of frozen, reheated akara were primarily governed by the texture and flavor of reheated product.

Comparison of recommended and nonrecommended processing conditions with other reheating methods by trained panel

To verify the optimum processing condition from the consumer test and compare different reheating methods, a trained panel test involving nine cooking treatments of akara was conducted. The treatments included two processing conditions from the optimum processing region (166°C ROT/75 sec ICT/90 sec RT, 166°C ROT/100 sec ICT/90 sec RT) identified from the consumer study and four from the nonoptimum region (149°C ROT/50 sec ICT/30 sec RT, 149°C ROT/50 sec ICT/60 sec RT,

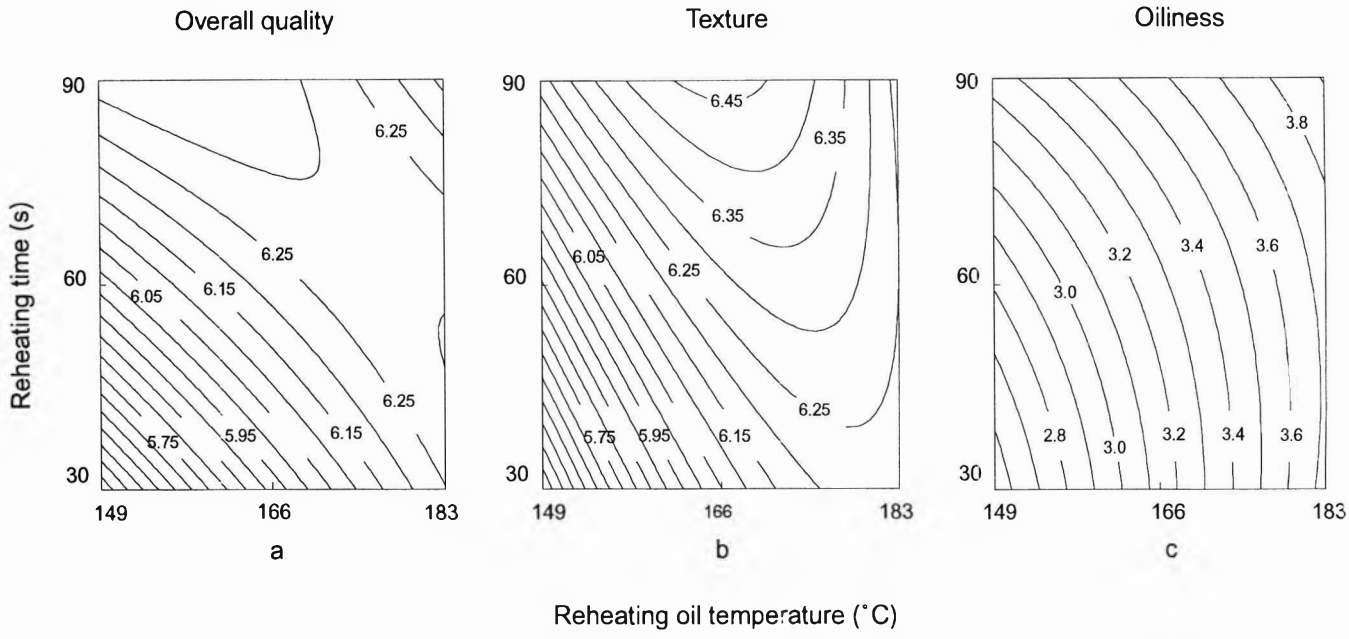

Fig. 1—Consumer responses of overall quality, texture and oiliness at 75 sec initial cooking time. Scales for overall quality and texture were 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely; scale for oiliness was 1 = extremely oily, 5 = just right, and 9 = extremely dry.

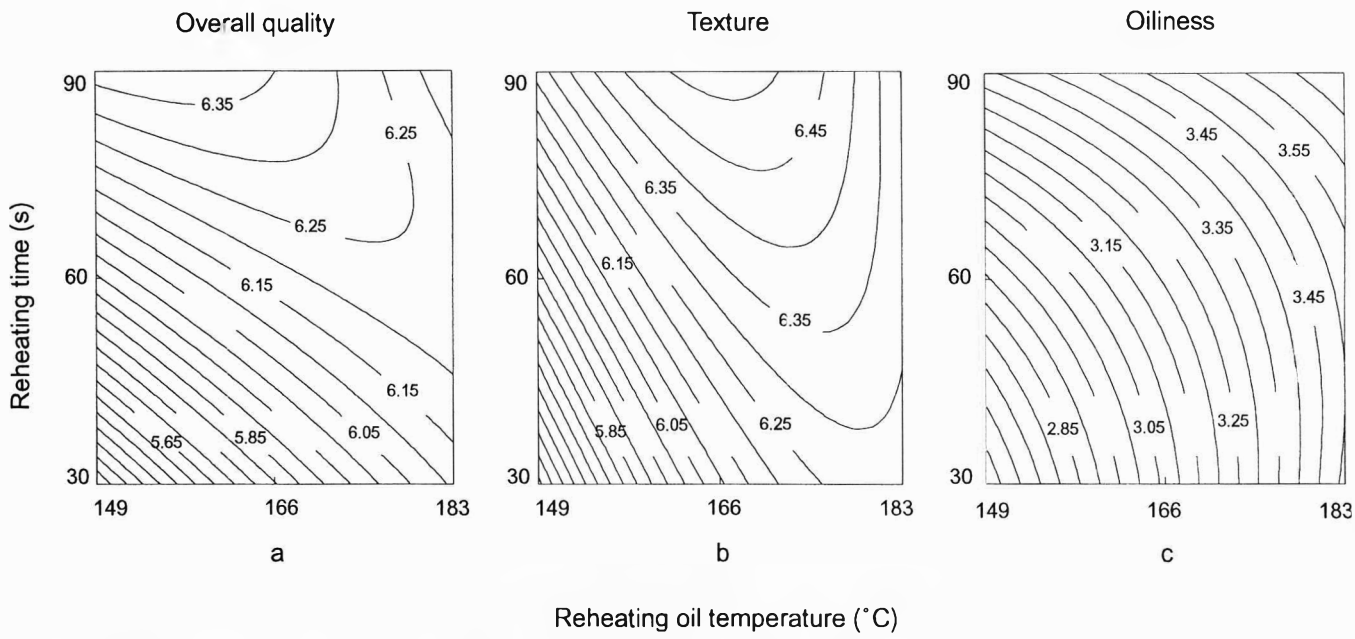

Fig. 2—Consumer responses of overall quality, texture and oiliness at 100 sec initial cooking time. Scales for overall quality and texture were 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely; scale for oiliness was 1 = extremely oily, 5 = just right, and 9 = extremely dry.

149°C ROT/100 sec ICT/30 sec RT, 183°C ROT/100 sec ICT/90 sec RT), plus freshly cooked akara, microwave-reheated akara, and conventional oven-reheated akara.

The results of trained panel analysis (Table 5) suggested that fresh akara and akara reheated by frying, baking, and microwaving were significantly different in color intensity, moistness, oiliness, crispness, and sponginess. Beany flavor was not affected by cooking conditions. No significant difference was found between one of the recommended frying reheating treatments (166°C ROT/75 sec ICT/90 sec RT) and freshly cooked akara for five sensory attributes. The other recommended frying reheating treatment identified from the consumer study, 166°C ROT/100 sec ICT/90 sec RT, was not different from fresh akara in all of the sensory attributes except crispness. This may have been due to the formation of a hard crust at the surface during reheating. Akara reheated at this condition was perceived as being more crisp and, therefore, more desirable than fresh akara. Comparison of the non-optimum frying reheating treatments with fresh akara indicated that all akara reheated at 149°C was paler than fresh akara, while that reheated at 183°C for 90 sec with a 100 sec initial cooking time was much darker (color intensity rating = 12.9). This color difference may be the reason that this treatment (183°C ROT/100 sec ICT/90 sec RT) was not included in the optimum processing region, although it was one of the treatments which received favorable responses for overall quality and willingness to purchase. Akara reheated at 149°C

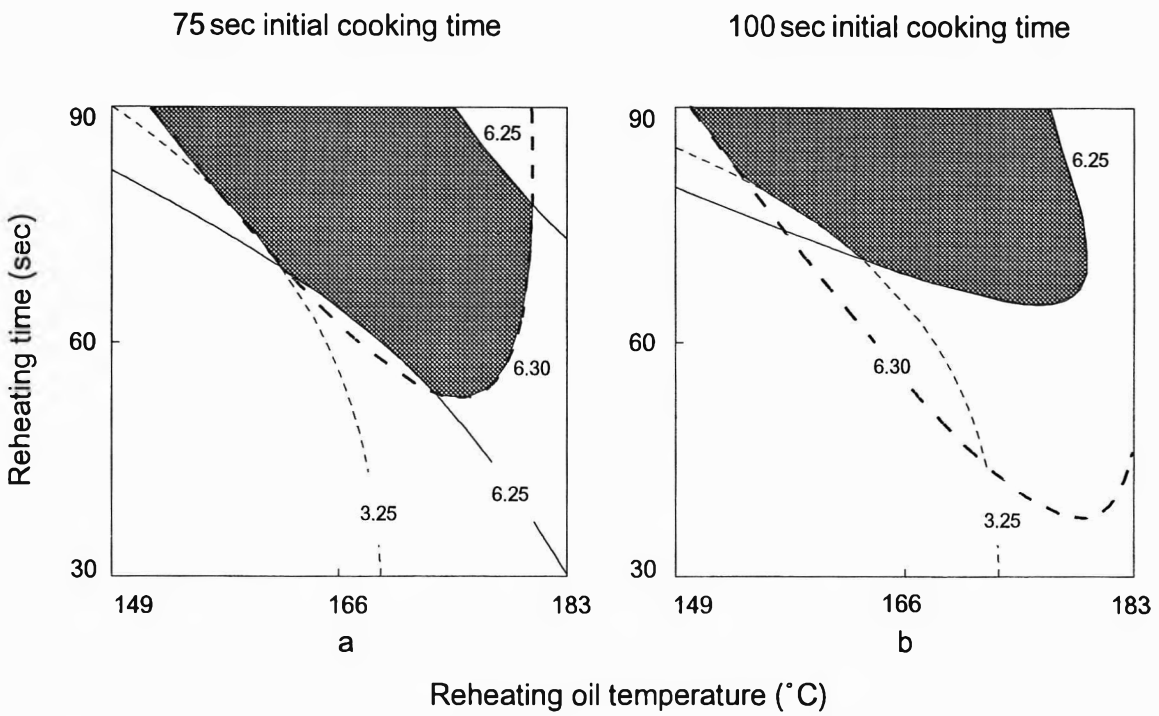

Fig. 3—Optimum regions from superimposed contour plots of consumer responses of overall quality, texture and oiliness at 75 and 100 sec initial cooking times. Scales for overall quality and texture were 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely; scale for oiliness was 1 = extremely oily, 5 = just right, and 9 = extremely dry. Overall quality ———, Texture ----, and Oiliness ·····.

Table 5—Comparison of sensory qualities of fresh akara and akara reheated by finish frying, microwaving and baking

Treatment	Color intensity[g]	Moistness[g]	Beany flavor[g]	Oiliness[g]	Crispness[g]	Sponginess[g]
Finish frying[f]						
149°C ROT/50 sec ICT/30 sec RT	7.8^{e}	6.8^{a}	6.0^{a}	9.4^{a}	5.3^{c}	9.4^{a}
149°C ROT/50 sec ICT/60 sec RT	7.9^{e}	6.1^{ab}	6.1^{a}	8.4^{ab}	5.4^{c}	8.9^{a}
149°C ROT/100 sec ICT/30 sec RT	8.5^{e}	5.9^{abc}	5.8^{a}	9.0^{ab}	5.4^{c}	9.4^{a}
*166°C ROT/75 sec ICT/90 sec RT	9.9^{d}	6.1^{ab}	5.4^{a}	7.9^{bc}	6.2^{bc}	9.3^{a}
*166°C ROT/100 sec ICT/90 sec RT	11.2^{bc}	5.7^{bc}	5.4^{a}	7.0^{cd}	6.8^{ab}	9.1^{a}
183°C ROT/100 sec ICT/90 sec RT	12.9^{a}	5.1^{cd}	5.2^{a}	6.3^{d}	7.7^{a}	9.0^{a}
Baking	10.4^{cd}	4.3^{d}	6.1^{a}	7.0^{cd}	6.0^{bc}	8.5^{ab}
Microwaving	11.9^{b}	5.0^{cd}	6.1^{a}	6.9^{cd}	5.4^{c}	7.8^{b}
Freshly prepared	10.6^{cd}	5.6^{bc}	7.7^{a}	7.0^{cd}	5.7^{c}	8.9^{a}

[a-e] Values followed by the same letter in a column are not significantly different ($P < 0.05$).
[f] ROT = Reheating oil temperature (°C), ICT = Initial cooking time (sec), RT = Reheating time (sec).
[g] Intensity ratings based on 15-cm unstructured line scales.
* Treatments selected from optimum region.

Table 6—Instrumental and chemical values for fresh akara and akara reheated by finish frying, microwaving, and baking

Treatment	Color			Moisture (%)	Fat content (%)		Shear force (N)	Puncture force (N)
	L	Chroma	Hue angle		Wet basis	Dry basis		
Finish frying[g]								
149°C ROT/50 sec ICT/30 sec RT	36.3^{a}	16.9^{b}	76.8^{a}	38.0^{b}	30.7^{b}	49.5^{a}	12.2^{f}	2.5^{c}
149°C ROT/50 sec ICT/60 sec RT	33.5^{b}	17.3^{b}	71.7^{b}	35.6^{c}	31.3^{b}	48.5^{a}	13.9^{ef}	2.8^{bc}
149°C ROT/100 sec ICT/30 sec RT	32.7^{b}	17.2^{b}	69.2^{bc}	35.1^{cd}	30.9^{b}	47.7^{a}	16.0^{e}	2.9^{bc}
*166°C ROT/75 sec ICT/90 sec RT	29.9^{cd}	17.5^{b}	68.1^{bc}	33.7^{cd}	32.3^{b}	48.7^{a}	25.3^{cd}	3.4^{ab}
*166°C ROT/100 sec ICT/90 sec RT	28.9^{d}	18.2^{b}	67.0^{c}	33.4^{d}	32.1^{b}	48.2^{a}	27.6^{bc}	3.4^{ab}
183°C ROT/100 sec ICT/90 sec RT	22.0^{e}	20.1^{a}	53.7^{d}	27.6^{f}	35.0^{a}	48.3^{a}	39.2^{a}	3.9^{a}
Baking	29.6^{cd}	17.8^{b}	69.1^{bc}	29.4^{ef}	22.5^{c}	32.0^{b}	29.1^{b}	2.9^{bc}
Microwaving	29.7^{cd}	17.6^{b}	67.6^{c}	30.6^{e}	23.0^{c}	33.1^{b}	22.4^{d}	2.6^{bc}
Freshly prepared	30.6^{c}	18.0^{b}	67.2^{c}	43.3^{a}	18.4^{d}	32.5^{b}	25.3^{cd}	3.2^{b}

[a-f] Values followed by the same letter in a column are not significantly different ($p < 0.05$).
[g] ROT = Reheating oil temperature (°C), ICT = Initial cooking time (sec), RT = Reheating time (sec).
* Treatment selected from optimum processing region.

was also more oily (higher oiliness intensity rating) than fresh product. This verified the consumer study and indicated that akara produced by the recommended conditions was better than that produced by non-recommended conditions.

Akara reheated by baking was dryer than fresh akara or products reheated by most frying conditions. This indicated that part of the moisture was removed during conventional oven reheating. To improve end product quality, adjustments should be

made in rehcating time and reheating oven temperature. The microwave-reheated akara was darker and less spongy than freshly cooked product. Adjustment in reheating power level and reheating conditions (with or without container) may enhance the quality of microwave-reheated products.

Instrumental/chemical measurements and correlation with trained panel analysis

Moisture, crude fat, texture and color data for fresh and reheated akara (treatments for trained panel test) were compared (Table 6). No significant difference was found between akara from selected processing conditions and fresh akara in chroma or hue angle. In general, akara cooked in higher temperature reheating oil with a longer cooking time (183°C ROT/100 sec ICT/90 sec RT) had a lower L value (darker), a smaller hue angle (browner) and higher chroma (more saturated) than akara reheated at lower oil temperature for shorter times (e.g., 149°C ROT/50 sec ICT/30 sec RT). There was no significant difference among baked, microwaved, or fresh akara in any instrumental color measurements. Sensory color intensity correlated with chroma, hue angle, and lightness (L value) resulting in an R^2 of 0.65, 0.69 and 0.82, respectively. The best fitting regression equation ($Y = 22.50 - 0.41X$, Y-sensory color intensity, X-L value) when results of all nine treatments were used for regression analysis had $R^2 = 0.82$.

The shear force values of akara from the two recommended treatments were not different from fresh akara. Akara reheated at a 149°C oil temperature (149°C ROT/50 sec ICT/30 sec RT, 149°C ROT/50 sec ICT/60 sec RT, and 149°C ROT/100 sec ICT/30 sec RT) was softer and akara reheated at a 183°C oil temperature (183°C ROT/100 sec ICT/90 sec RT) was harder than fresh products. Puncture force was measured as the force (N) of a probe required to puncture through the crust of akara. No difference was found between the two recommended treatments (Table 6) and fresh akara. Since the color readings of akara from the recommended treatments were also not different from fresh akara, this also indicated that the quality of akara from recommended conditions was closer to fresh akara than that from non-recommended conditions. Sensory crispness and puncture force were correlated by using a linear regression analysis ($Y = 0.92 + 1.66X$; Y-sensory crispness rating, X-puncture force). An $R^2 = 0.86$ was obtained using all nine treatments.

The moisture and crude fat contents (both dry basis and wet basis) of akara from the two selected conditions were different from those of fresh akara, while no difference was found in oiliness sensation by the trained panel. When panelists were tasting a fried product, the oiliness attribute was the most difficult to assess since sensations of moistness and oiliness interacted. Sensory oiliness correlated poorly with crude fat content ($R^2 = 0.14$), however, a linear regression including fresh akara and all six frying reheating treatments between sensory oiliness and dry basis crude fat and moisture content resulted in $R^2 = 0.84$. By using this regression equation, $Y = -13.22 + 0.23X1 + 0.29X2$ (Y-sensory oiliness; X1-dry basis crude fat; X2-moisture content), the predicted sensory oiliness rating for fresh akara was 6.8, very close to the actual mean sensory rating of 7.0. Consumer test results showed akara reheated at higher oil temperature for a longer cooking time had a relatively higher oiliness rating (less oily). Thus, at a given crude fat content, akara with a higher moisture content may be perceived to be more oily than that with a lower moisture content.

Instrumental/chemical measurements supported the trained panel results that akara reheated by baking was dryer than fresh akara or akara from most other frying/reheating conditions. Akara reheated by baking was also harder (higher shear force) than fresh akara. However, one advantage of baking or microwaving over the frying reheating conditions would be lower fat content.

CONCLUSIONS

AKARA HAS POTENTIAL to be marketed as a frozen, ready to reheat product. By using frying for reheating, initial cooking time should be >50 sec. With 75 sec initial cooking, reheating time should be ≥50 sec, while the reheating oil temperature could range from about 152°C to 180°C. For 100 sec initial cook time, the reheating time should be >70 sec and reheating oil temperature not exceed 180°C. Sensory and physical properties of akara from selected processing conditions were close to those of fresh akara, while akara from non-recommended processing conditions was different from fresh product. Shear force of akara reheated by baking was higher than that of fresh product. Akara reheated by microwaving was less spongy than fresh akara. Sensory color intensity correlated with lightness, crispness correlated with puncture force, and oiliness correlated with crude fat content together with moisture content.

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Ms received 12/29/94; revised 4/28/95; accepted 5/20/95.

This work was supported by state and Hatch funds allocated to the Georgia Agricultural Experiment Stations and by a grant from the Bean/Cowpea Collaborative Research Support Program, U.S. Agency for International Development.

We are grateful to Julia Heggie, Laura Milius, Richard Stinchcomb, and Bob Flewellen for technical assistance.

Physicochemical Properties of Rice Steeped in Hot or Warm Water

AIJIRO YAMAMOTO

ABSTRACT

Prolonged steeping of rice grains in hot or warm water was examined to study the gelatinization process of rice starch. Rice treated at 75°C for 8–18 hr showed the same type "V" X-ray diffraction pattern as that of cooked rice. Rice treated at 50–60°C for 24–72 hr showed a strengthened type "A" pattern. The gelatinization degree of the 75°C treated rice determined by the β-amylase-pullulanase method and Brabender amylography was as high as that of cooked rice. The value of the 50–60°C treated rice was lower than that of raw rice. The gelatinization temperature of rice varied with the two types of treatment.

Key Words: rice, gelatinization, starch granules, amylase, hydrolysis

INTRODUCTION

"WARM WATER TREATMENT" (Gough and Phbus, 1971) and "heat moisture treatment" (Lorenz and Kulp, 1982) cause physical modifications of starch or annealing of starch granules (Kuge and Kitamura, 1985). The effects of "warm water treatment" on starch granules have been extensively studied in wheat grains (Gough and Phbus, 1971), as well as starches such as potato (Yamamoto et al., 1983) and other isolated starches. Warm water treatment changes starch granules to a thermodynamically more stable state, which probably results from the realignment of the polymer chains in crystalline and amorphous regions, and an increase in homogeneity of starch granules (Kuge et al., 1985). However, such a study has not been reported on whole rice grains or isolated rice starch granules. Spoiled rice resulting from inappropriate cooking (commonly called "Mekko-meshi" in Japan) has been explained as an effect of warm water treatment. Such rice is usually produced after disruption of heating in early cooking in a traditional kitchen range. The abnormally cooked rice thus obtained has undesirable mouth-feel and the texture is not improved after re-cooking.

Our objective was to study the effects of prolonged steeping in hot or warm water on the physicochemical properties of rice grains to elucidate the gelatinization process at 50–75°C and the mechanism of production of abnormally cooked rice.

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MATERIALS & METHODS

Rice

Koshihikari, one of the typical cultivars of *japonica* type grown in Niigata Prefecture in 1988, Yukihikari, one of the cultivars of *japonica* type with a slightly higher amylose content grown in Hokkaido in 1988, and Hoshiyutaka, a cultivar of *indica* type grown in Okayama Prefecture in 1988 were commercially obtained as polished rice. All rice species were packed in laminated film bags of nylon and polyethylene and then stored up to 4 yr at 5°C in a refrigerator.

Preparation of rice

Raw rice specimen was prepared by grinding raw polished rice grains, and screening the flour through a 60 mesh sieve by a centrifugal mill. Cooked samples were prepared by cooking 200g of rice grains in 300 mL water, using an automatic electric cooker (National SR-3060, 0.6L or Sharp KS-05T2, 0.54L) for 20 min at up to 100°C. Hot water treated rice (HWR) was prepared by steeping 200g of rice in 300 mL (or 800 mL) water at a constant 75°C in an automatic electric cooker (Sharp KS-

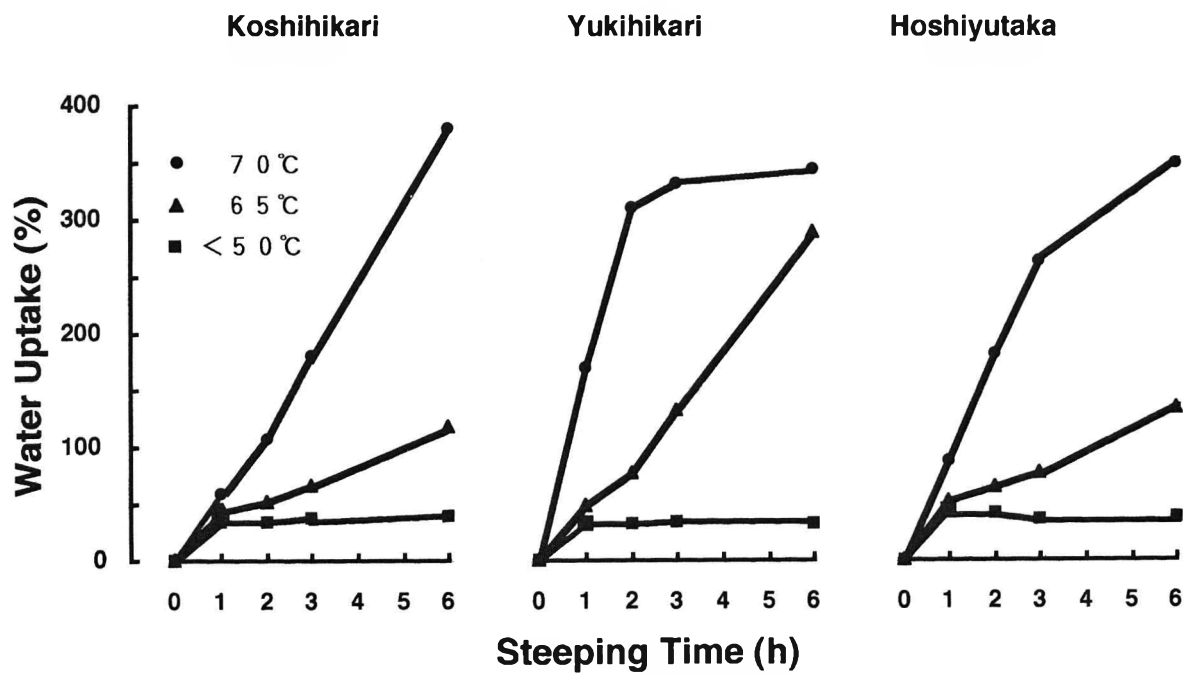

Fig. 1—Water uptake of polished rice at various temperatures.

Table 1—Texturometer measurements of HWR (Yukihikari)

	Hardness[a] (H)	Stickiness[a] (−H)	Palatability[a] (−H/H)
Water/grain: 1.2 (w/w)			
Cooked rice	3.03 ± .18	0.40 ± .11	0.134
HWR			
upper	4.04 ± .10	0.01 ± .01	0.003
middle	3.22 ± .22[b]	0.27 ± .05[b]	0.085[b]
bottom	2.94 ± .07[ce]	0.57 ± .03[cd]	0.194[cd]
Water/grain: 1.5 (w/w)			
Cooked rice	2.56 ± .20	0.71 ± .12	0.28
HWR			
upper	3.72 ± .33	0.07 ± .05	0.019
middle	2.93 ± .43[b]	0.42 ± .11[b]	0.148[b]
bottom	2.62 ± .22[c]	0.67 ± .04[cd]	0.257[cd]

[a] Mean value ± standard deviation (n = 6).
[b,c] significantly diferent from upper at $P < 0.01$.
[d] significantly different from middle at $P < 0.01$.
[e] significantly different from middle at $P < 0.05$.

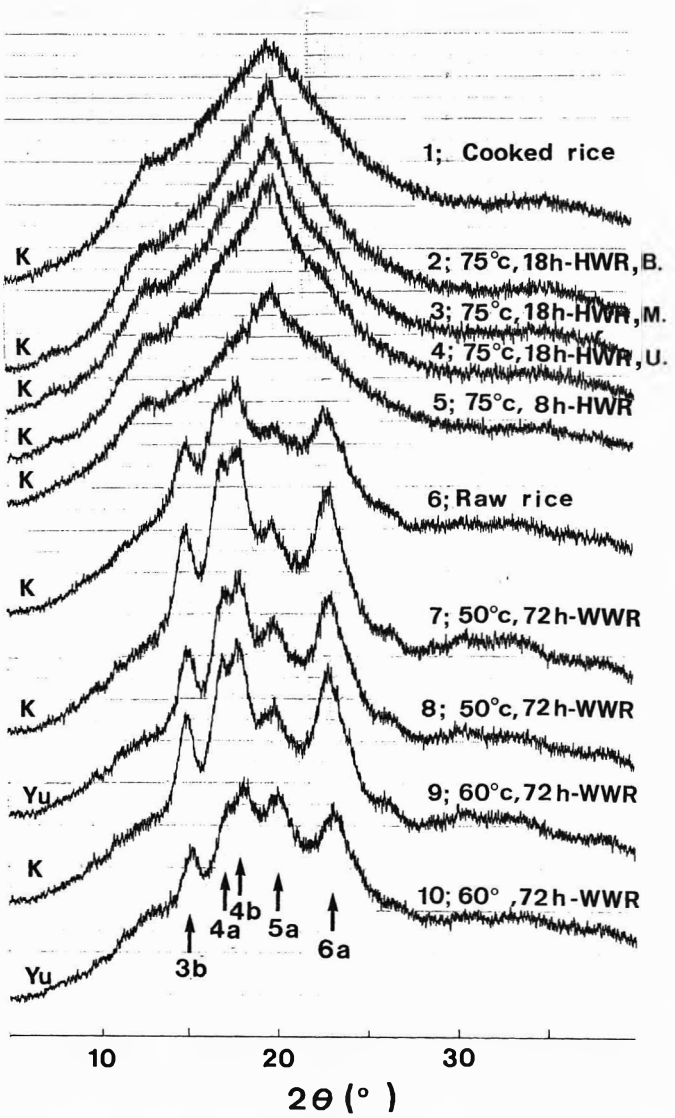

Fig. 2—X-ray diffraction intensity diagrams of cooked rice, HWR, raw rice, and WWR.

05T2, 0.54L) for 8, 12 and 18 hr. Pressure cooked rice was prepared by cooking 200g of rice grains in 300 mL water from 1 to 1.2 kg/cm² in a pressure cooker (Syowa matsutaka, 1.8L) for 5 to 10 min with a gas heater. Warm water treated rice (WWR) was prepared by steeping 200g rice in 1L water at 50 or 60°C in a water bath for 24 or 72 hr. Alkali-treated rice was prepared by mixing 200g raw rice flour with 600 mL water and 600 mL 2N NaOH. The mixture was kept at 50°C in a water bath for 15 min, then neutralized with 600 mL 2N acetic acid. Aged rice was prepared by storing cooked rice at 5°C for 48 hr and 96 hr. Three commercially cooked rice specimens (A, B and C) were also obtained from a university cafeteria and a local store, and their gelatinization degree (GD) was checked. All rice specimens were homogenized in a Waring blender and rapidly dehydrated with ethanol and ether according as described by Mitsuda et al. (1983) to prevent further change of the GD. Cooked rice samples for texture analysis were obtained by steeping 7 hr at room temperature and then cooking in an automatic electric cooker, while HWR was steeped at 65°C for 7 hr and then cooked. Rice specimens were separated into the upper, middle and bottom portions of a cooking vessel.

Table 2—Gelatinization degree of rice determined by the β-amylase-pullulanase method (calculated as percentage of the alkali gelatinized specimen)

Rice samples	Gelatinization degree (%)
Koshihikari	
Cooked rice	
just after cooking	85.1
aged at 5°C for 96 hr	55.7
Raw rice	28.2
WWR	
50°C for 72 hr	23.4
60°C for 72 hr	22.1
HWR	
75°C for 18 hr (upper)	89.3
(middle)	89.4
(bottom)	80.0
8 hr (mixed)	89.0
4 hr (mixed)	52.1
(bottom)	47.9
Yukihikari	
Raw rice	26.9
WWR	
50°C for 72 hr	20.6
60°C for 72 hr	48.4

Table 3—Characteristics of amylogram of three cultivars of raw rice

Characteristics		Koshihikari[a]	Yukihikari[a]	Hoshiyutaka[a]
Gela. Temp.	(°C)	69.0 ± 5.5	67.3 ± 4.6	66.4 ± 1.0
Max. Visco.	(BU)	384.7 ± 8.7	255.5 ± 2.9	455.7 ± 13.5
Max. Temp.	(°C)	94.8 ± 0.7	94.5 ± 1.2	95.0 ± 2.6
Hot Paste Visco.	(BU)	225.3 ± 7.0	166.3 ± 4.2	287.8 ± 9.2
Break down	(BU)	159.3 ± 9.7	88.8 ± 7.1	167.9 ± 6.4
Final Visco.	(BU)	439.8 ± 8.6	390.0 ± 15.1	555.9 ± 10.9
Consistency	(BU)	214.5 ± 13.7	223.7 ± 13.8	268.1 ± 7.6
Bre./Con.		0.743 ± 0.02	0.400 ± 0.05	0.626 ± 0.02

[a] Mean value ± standard deviation (n = 3 ~ 5)

Analytical methods

Water uptake test of polished rice was done at 25–70°C. The texturometric characteristics of cooked rice and hot water treated rice specimens were determined as described by Mitsuda et al. (1983).

The GD of rice specimens was determined by the β-amylase-pullulanase method of Kainuma et al. (1981) to examine effects of treatments on gelatinization.

The X-ray diffraction pattern was observed for rice specimens and used to determine the state of crystalline structure of starch. A Shimadzu X-ray diffraction autoanalyzer, XD-3A model, was used. Operating conditions were: X-ray generator, Cu-Kα-ray excited at 40 kV; tube current 30 mA; rate meter, fixed; generator slit, 0.6 mm; slit for protection of air scattering, 1°; photo-accepter slit 0.3 mm; scanning speed of the geniometer, 1°/min; time constant, 1.0 sec; and chart speed 10 mm/min. Peaks in the X-ray diffraction pattern were named according to Hizukuri and Nikuni (1957).

The rheological characteristics of rice specimens were determined with a Brabender amylograph on an 8% paste. The amylograph characteristics were gelatinization temperature, maximum viscosity, temperature at maximum viscosity, hot paste viscosity, break-down, final viscosity at 50°C, consistency, and break-down per consistency. Determination was repeated three to seven times for each sample and averages and standard deviations were calculated.

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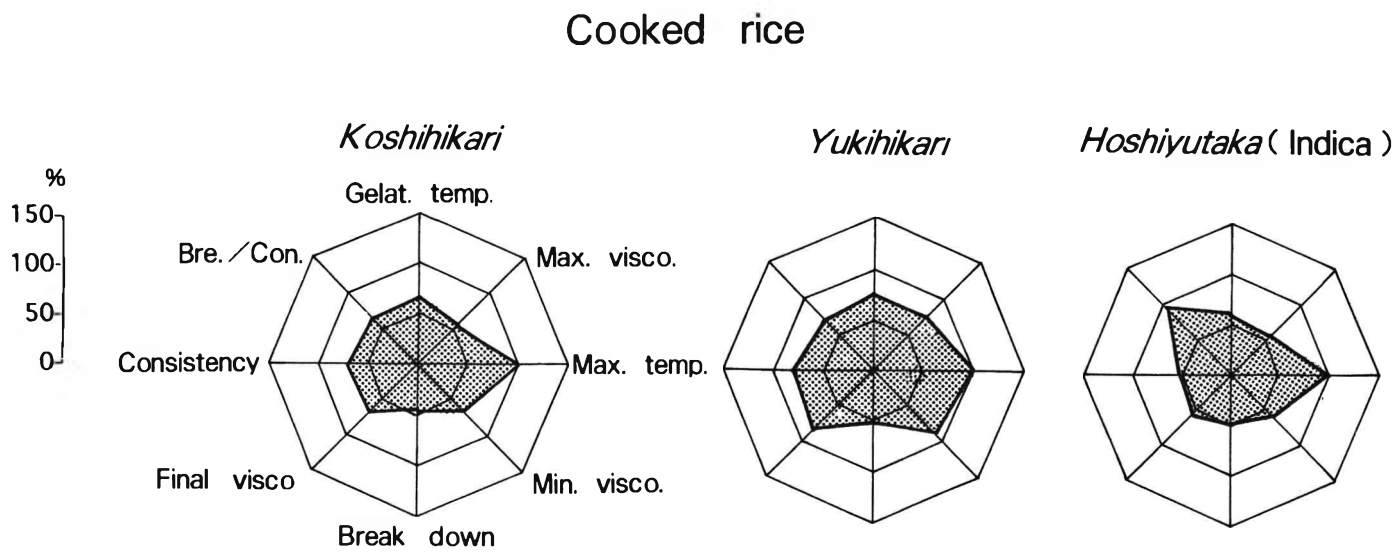

Fig. 3—Polar diagrams of cooked rice. Small, middle and large circles indicate 50, 100 and 150% of the values of eight characteristics of raw rice.

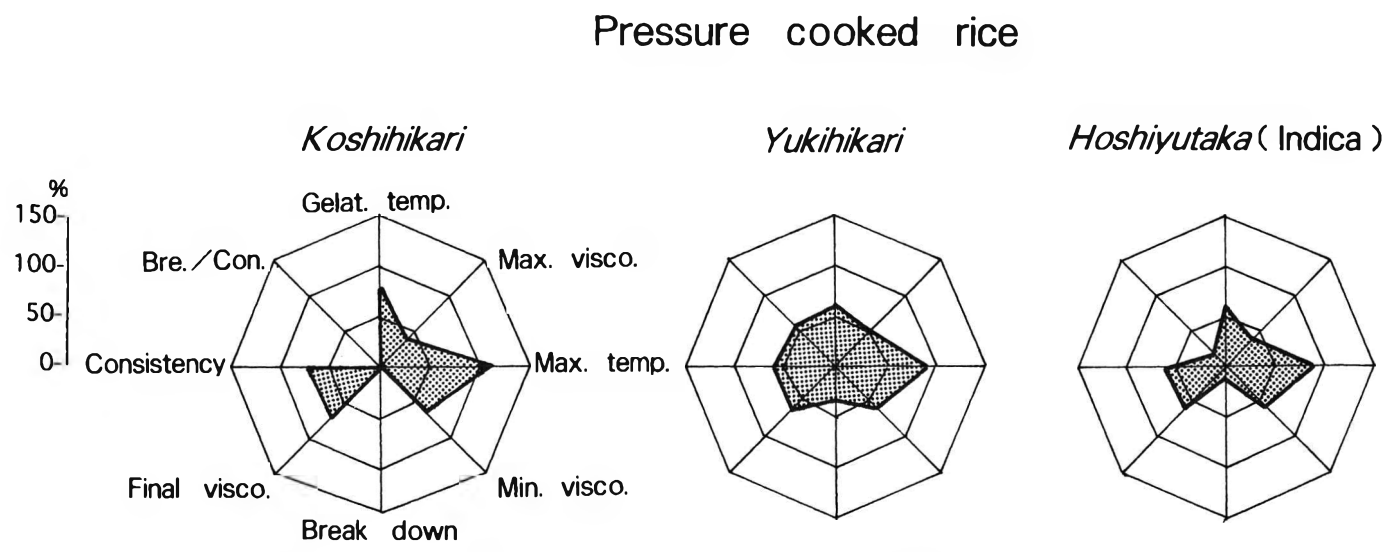

Fig. 4—Polar diagrams of pressure-cooked rice. The same diagrams as in Fig. 3 were used.

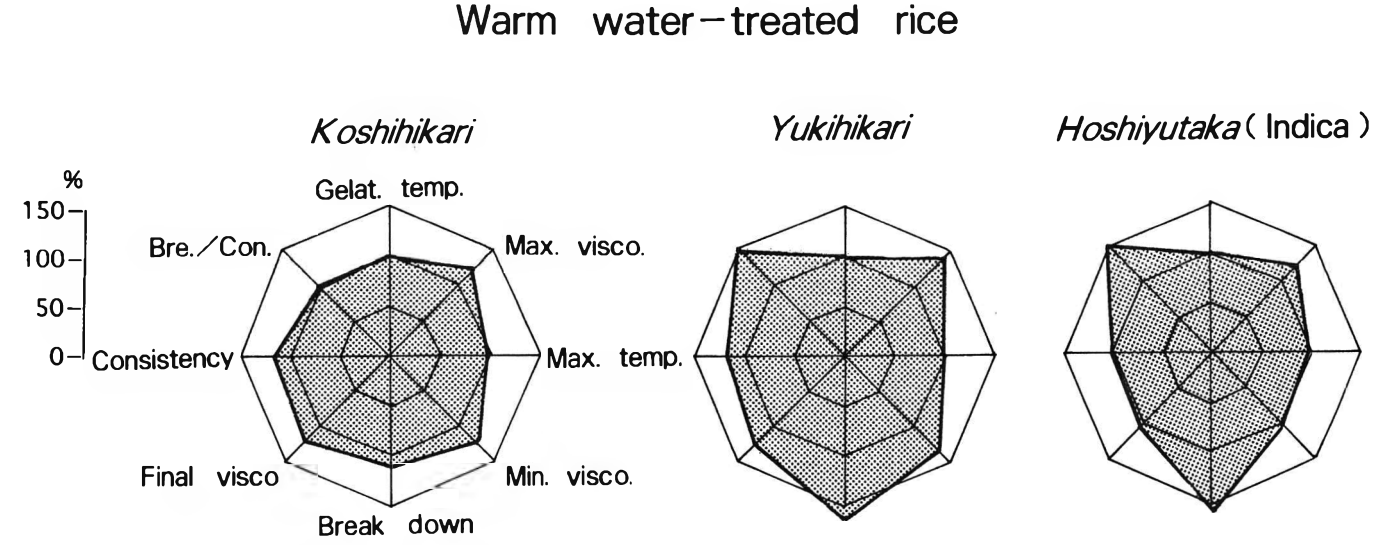

Fig. 5—Polar diagrams of WWR. The same diagrams as in Fig. 3 were used.

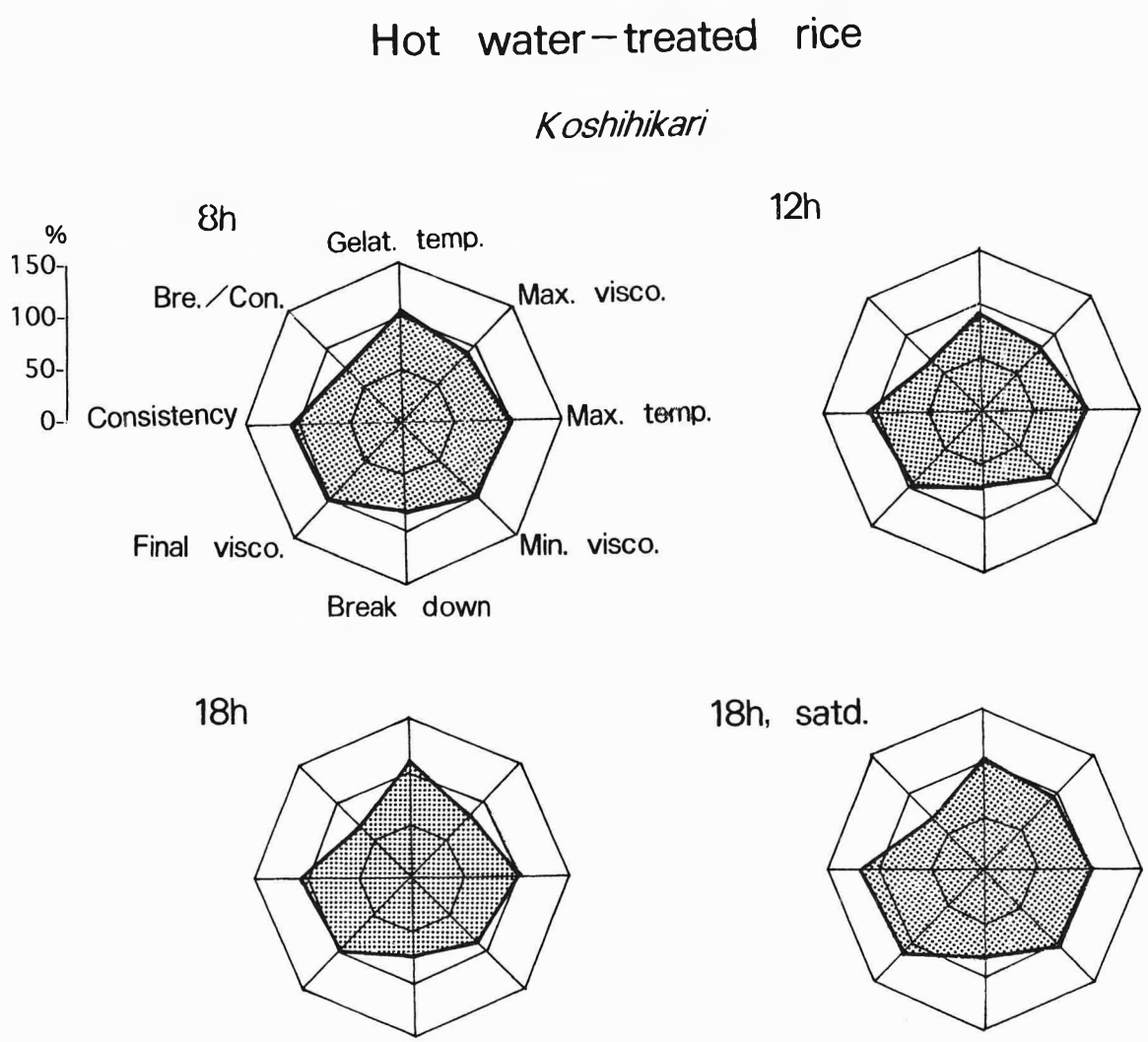

Fig. 6—Polar diagrams of HWR. The same diagrams as in Fig. 3 were used.

RESULTS & DISCUSSION

Water uptake of polished rice at various temperatures

The time course was recorded for the water uptake of polished rice (Fig. 1). The weight changes of three cultivars of rice were tested at various temperatures for 6 hr. At 50°C, water uptake reached 30% of original rice weight. Water uptake was further increased at 65°C, reaching 300%. At 70°C, the water uptake of Yukihikari increased rapidly and equilibrated after 3 hr steeping. That of Koshihikari and Hoshiyutaka increased slowly and did not reach equilibrium even after 3 hr. This difference may have been due to differences of gelatinization temperatures of these cultivars. Gelatinization temperature of Yukihikari was the lowest.

Texture of cooked rice and HWR

Texturometer measurements were compared for cooked rice and HWR after steeping and cooking (Table 1). At water to grain ratios of 1.2 and 1.5, all values of HWR in the upper, middle and bottom portions were significantly different. In both ratios, HWR was hard and less sticky in the upper portion and soft and more sticky in the bottom portion. HWR had undesirable mouth-feel like raw rice paste and heterogeneous texture in different parts of the utensil. HWR was cooked again after addition of 1/10 volume of water, but the texture of rice did not improve.

X-ray diffraction

X-ray diffraction intensity diagrams were compared for cooked rice, HWR, raw rice, and WWR of Koshihikari and Yukihikari (Fig. 2). Lines 6 to 10 represent a typical type "A" (raw cereal type) pattern (Hizukuri and Nikuni, 1957). The peak heights of WWR, which had been kept at 50°C and 60°C for 72 hr, were larger than those of raw rice. This indicated that the crystalline degree of the 50–60°C treated rice was stronger than that of the raw rice. This confirmed the hypothesis that a rearrangement of the crystalline structure of the rice starch could occur during the steeping (Yamamoto et al., 1983). However, the peak of Yukihikari was lower at 60°C (line 10) than at 50°C (line 8), which indicated that partial gelatinization occurred in that cultivar. On the contrary, the bottom (line 2), middle (line 3) and upper (line 4) portions of HWR obtained by an 18h-treatment showed a type "V" (gelatinized type) pattern similar to that of cooked rice (line 1) with only one peak at 5a. A small peak (3b) remained in the upper (line 4) portion of HWR. The mixture of HWR obtained by an 8 hr treatment (line 5) also showed a type "V" pattern. A small peak at around 13° was derived from the helix structure of nonglutinous rice starch.

Changes in the X-ray diffraction intensity diagram (data not shown) were noted in aged rice and HWR after 4 hr treatment. HWR showed the transit pattern from type "A" to type "V". A similar transit pattern was observed in aged rice, although the intensity of each peak was slightly different from that of HWR. The 3b peak was low in aged rice, while the 3b and 6a peaks

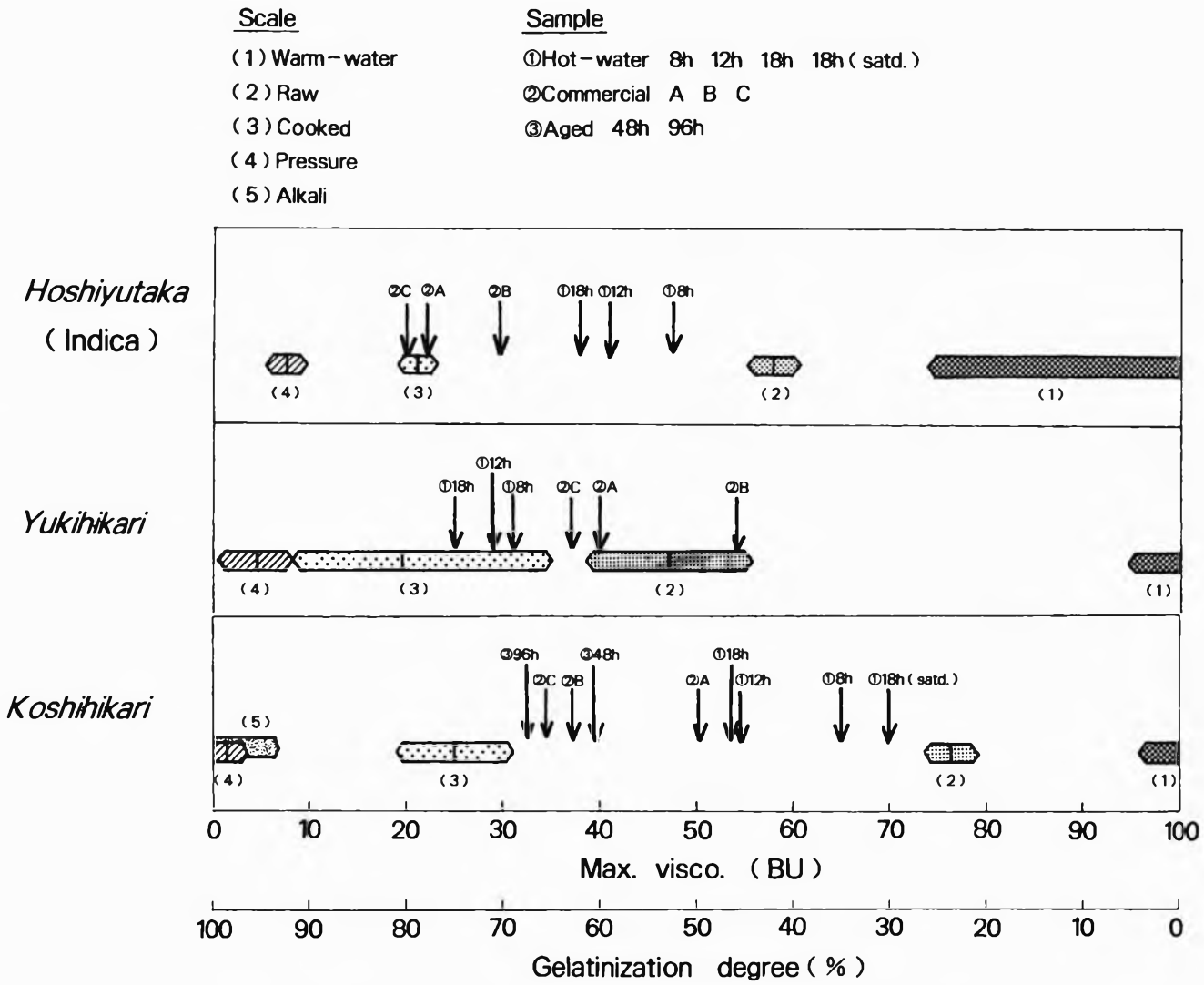

Fig. 7—Estimation of the GD of rice specimens using the scale of maximum viscosity. Hatched bars show the average values of viscosity for five specimens. (1) to (5) with ranges of possibilities. Arrows indicate average values of viscosity for specimens such as HWR, aged rice and commercially cooked rice.

were high in HWR. The pattern of aged rice may indicate the transit from type "V" to type "B" (raw tuber type) pattern, reported by Hibi et al. (1986).

GD determined by β-amylase-pullulanase method

GD values of raw rice, cooked rice, aged rice, WWR, and HWR were compared by the β-amylase-pullulanase method (Table 2). The GD of HWR was as high as that of cooked rice, while the value for WWR was lower than that of raw rice. The warm water treatment changes starch granules to a thermodynamically more stable state, which probably resulted from the realignment of polymer chains in both crystalline and amorphous regions and an increase in homogeneity of the starch granules (Kuge et al., 1985). We assumed that raw polished rice in Japan was partially gelatinized by its hysteresis such as heat-drying of paddy rice, transportation and storage of brown rice in a hot ambient temperature, and friction heat during polishing. Thus, the lowest point of the GD of raw polished rice could not be estimated.

GD determined by Brabender amylography

Characteristics of the amylogram of three cultivars of raw rice were compared (Table 3). The polar diagrams of eight characteristics of cooked rice were compared, in which small, middle and large circles indicated 50, 100 and 150% of raw rice (Fig. 3). Similar diagrams were also compared for the other treatments. For each cultivar the maximum viscosity and breakdown decreased greatly. Generally, the maximum viscosity represents starch granules that do not swell and rupture. Break down indicates the rupture of such granules. Decrease of these characteristics by cooking implies that the GD of cooked rice was higher than the GD of raw rice. In pressure cooked rice (Fig. 4) break-down decreased to 0, 33 and 10% in Koshihikari, Yukihikari, and Hoshiyutaka, respectively. However, in all cultivars, maximum viscosity of pressure-cooked rice remained at 40 to 50% that of raw rice. Alkali-treated rice also indicated no break down and 37% maximum viscosity (data not shown). Higher values in maximum viscosity reflect higher viscosity of such specimens at room temperature. For WWR all viscosities were higher than 100% (Fig. 5) which implied that the GD of WWR was lower than those of cooked, pressure-cooked or raw rice. Therefore we substituted WWR for raw rice as the least gelatinized (most crystalline) state of starch.

In contrast, the maximum viscosity and break down decreased slightly in HWR after 8, 12 and 18h (Fig. 6, Koshihikari). This indicates that the GD of HWR was slightly higher than that of the raw rice and much lower than that of cooked and pressure-cooked rice. The maximum viscosity and break-down did not

decrease so much even in HWR after steeping in 4 volumes of hot water for 18 hr (Koshihikari, 18h, satd.). Thus the cause of the low GD of HWR was assumed to be lack of sufficient temperature rather than lack of water. Similar results were found for HWR of Yukihikari and Hoshiyutaka. A positive correlation was found between maximum viscosity and break-down for the 31 samples of three cultivars on the amylograph;

$$\text{(Maximum viscosity)} = 1.7 \times \text{(break down)} + 124, \quad r = 0.98$$

where r is the correlation coefficient. The maximum viscosity in the Brabender amylogram was proportional to the GD of the rice specimen. A scale was proposed (Fig. 7) on which the GD of rice specimens was estimated by the maximum viscosity values. Each specimen was assumed to be located at a reasonable position on the scale.

We examined the GD by X-ray diffraction, β-amylase-pullulanase and amylography, because of the lack of a critical method to determine GD. Our results indicated that the X-ray diffraction pattern analysis could clearly distinguish the type "A" pattern of WWR and type "V" pattern of HWR. The GD was assumed from the peak heights of each pattern, but quantitative values could not be obtained.

HWR kept at 75°C for more than 8 hr showed a high GD, similar to the GD of cooked rice while the GD of WWR kept at 50°C or 60°C for 72 hr was lower than the GD of raw rice. The GD determined by the β-amylase-pullulanase method appeared to be in agreement with results by X-ray diffraction pattern and with the water uptake behavior of rice grains.

By amylography, the GD of HWR was estimated to be 30–40%, while the GD of cooked rice was 67–75%. For the GD of the HWR and cooked rice, values estimated by amylography were lower than those determined by the β-amylase-pullulanase method. This may be because the enzyme method showed a susceptibility of starch to enzyme attack while amylography showed the presence of starch granules that did not swell in the endosperm. The lack of edibility because of texture was explained by amylography better than by X-ray diffraction analysis and β-amylase-pullulanase methods.

In HWR, the crystalline structure was assumed to be almost completely broken down and the starch partially susceptible to enzyme hydrolysis. However, some starch granules did not swell in the endosperm and remained to a considerable extent even when there was sufficient water for absorption at <75°C. Even in the cooked rice, starch granules in the endosperm remained intact in contrast to pressure-cooked rice and alkali-treated rice. These results were in agreement with the finding that pressure-cooked rice was more sticky than conventionally cooked rice. Kato et al (1981) reported that starch granules did not fully swell even after heating at 100°C for 20 min. Similar results were reported in the boiling of noodles by Saito (1980).

In WWR, the crystalline structure was assumed to be stronger than that of raw rice. Susceptibility to enzyme hydrolysis decreased and the number of starch granules that did not swell increased even after steeping in sufficient water at 50–60°C for 24–72 hr. This could be explained by the rearrangement of the crystalline structure of rice starch. Further studies are needed to determine the relative effects of rice crystalline structure rearrangements.

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Ms received 9/23/94; revised 5/5/95; accepted 6/22/95.

This study was supported in part by Special Research Grant (1993) from Koshien University.

The author is grateful to K. Ishibashi, E. Kakemizu, H. Sakae, and M. Kobayashi for technical assistance.

Heat Denaturation of Durum Wheat Semolina β-Amylase Effects of Chemical Factors and Pasta Processing Conditions

MARIE-FRANÇOISE SAMSON and MARIE-HÉLÈNE MOREL

ABSTRACT

The susceptibility to heat denaturation of durum wheat β-amylase was studied in aqueous solution at 50 to 65°C. The activation energy of the reaction of denaturation was 439 kJ/mole between 56 and 65°C. Additives were tested to determine their protective effects on enzyme activity (at 65°C) in aqueous solution. Maltose, resulting from degradation of starch by β-amylase, appeared to be the most protective. The β-amylase became more resistant to heat with higher concentrations of maltose, indicating an enzyme-maltose complex which was more stable to heat than the enzyme alone. The same mechanism occurred when durum wheat pasta was processed: maltose produced either during mixing and sheeting or during extrusion protected the enzyme. The degree of protection was proportional to the intensity of mechanical work imparted to the dough.

Key Words: durum wheat, semolina, beta-amylase, pasta processing, heat denaturation

INTRODUCTION

AMONG THE ENZYMES of durum wheat semolina, those most extensively studied directly affect color and cooking quality of spaghetti (Feillet, 1987). Lipoxygenases are specific for the oxidation of fatty acids. They are important to pasta color and are responsible for the yellow index. This index depends on the presence of carotenoïd pigments which are oxidized by lipoxygenases. Although varietal differences may exist, the action of such enzymes remains limited if any contamination by the lipoxygenase-rich germ is avoided. The activity of such enzymes can be prevented by mixing and extrusion under vacuum and by application of high temperatures at some stages of drying (Abecassis, 1991).

Peroxidases and polyphenol oxidases are also of major importance in determination of the color of finished products, particularly brown index which is highly correlated with activity of peroxidases. However, since the introduction of pasta drying at high temperatures, color was improved due to inactivation of peroxidase and polyphenol oxidase activities (Kobrehel and Abecassis, 1985).

The effect of α-amylases on pasta quality is controversial, particularly regarding pasta made of germinated wheat (Feillet, 1987). These enzymes hydrolyze α-(1 → 4) glycosidic bonds of starch and derived polysaccharides at random. They are virtually absent from mature grain as they are synthesized mainly during germination. For this reason, studies of durum wheat α-amylases were associated with those aimed at following the effects of sprouted durum wheat on pasta quality. According to D'Appolonia (1981) and Kruger and Matsuo (1982), the use of sprouted wheats in pasta production induced more solids losses in cooking water. Simpler sugars (maltose, glucose) and high molecular weight dextrins are leached resulting in pasta stickiness and poor surface quality of the cooked pasta. In contrast, according to Matsuo et al. (1982) and Combe et al. (1988), pasta quality is lowered by the use of germinated wheats.

The effects of β-amylase (α-1,4-glucan maltohydrolase; EC 3.2.1.2.) are not as widely known as those of the above-mentioned enzymes. It catalyses the liberation of β-maltose from the nonreducing ends of starch and related 1,4-α-glucans. Most knowledge about this enzyme was acquired through studies of barley and bread wheat, and is essentially related to its genetic polymorphism (Joudrier and Bernard, 1977; Joudrier and Gobin, 1982; Ainsworth et al., 1983) or biochemical polymorphism (Nummi et al., 1965; Niku-Paavola et al., 1973; Lundgard and Svensson, 1987; Bureau et al., 1989; Gupta et al., 1991). The physiology of β-amylase was investigated during grain development (Kruger, 1972; Nishimura et al., 1987; Laurière et al., 1986) or germination (Daussant and Corvazier, 1970; Sopanen and Laurière, 1989; Bureau et al., 1989; Guerin et al., 1992). Both α- and β-amylases are starch degrading enzymes, but α-amylase is synthesized during germination whereas β-amylase is synthesized during development and stored into endosperm until germination (Kruger, 1972; Nishimura et al., 1987; Laurière et al., 1986). There are two forms of β-amylase, a free one and an inactive one bound to other proteins, particularly glutenins (Rowsell and Goad, 1962a, b; Gupta et al., 1991; Lew et al., 1992; Werner et al., 1992). Several researchers have assumed that β-amylase may act as a storage protein (Giese and Hejgaard, 1984; Gupta et al., 1991).

Industrial pasta has been processed from increasingly fine semolina and using higher extrusion rates (Manser, 1985) to increase productivity. Also, the pasta drying temperature was raised from 55°C to 90°C or more. The main disadvantage has been a red color development for dried pasta, as a consequence of an increase of Maillard reactions. This suggested reconsideration of the importance of β-amylase in pasta quality. First, β-amylase reacts with the starch granules damaged during milling to produce maltose and dextrins. These are probably responsible for cooking losses and stickiness, as in the case of sprouted wheat (D'Appolonia, 1981; Kruger and Matsuo, 1982), which could also induce the development of a red color for pasta dried at high temperatures. Second, because flours contain much more damaged starch than semolinas, the trend to use finer semolina is likely to favor β-amylolysis in pasta. Third, higher mixing and extrusion speeds should favor amylolysis by increasing enzyme-substrate contacts. However, intensified mechanical work also causes starch damage and increases dough temperature which may have a deleterious effect on the enzyme. While α-amylase is highly resistant to heat, β-amylase is susceptible to high temperatures and its maximum activity is reached at 50°C (Meyer et al., 1953).

Our objective was to determine the influence of both heat denaturation and mechanical work on the activity of β-amylase. Heat denaturation of the enzyme was first studied in water to explore the reaction in a simple medium (activation energy of denaturation, protective effect of additives). Then, the study was carried out on dough to assess the effects of mechanical treatment imparted to the dough during pasta processing on β-amylase physiochemical properties. Effects on pasta quality were not investigated.

MATERIALS & METHODS

Durum wheat semolina

Durum wheat (*Triticum durum* Desf.) semolina was prepared from cultivar Ardente in a Bülher MLU 202 mill with 2.5 kg grains. The milling diagram indicated a yield of ≈ 60%. The semolina protein con-

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tent was 15.1% and its moisture level 15.8%. Semolina was stored at +4°C.

Chemicals

All reagents were of analytical grade. Sodium chloride, bovine serum albumin, glucose, monohydrated maltose, soluble starch according to Zulkowsky, sucrose, monohydrated lactose, polyethylene glycol 300 and 20,000, and ethylene glycol were purchased from Merck (Darmstadt, Germany); enzymes were purchased from Boehringer (Mannheim, Germany) and Sigma (St Louis, USA). Industrial gluten was kindly supplied by the firm Roquette Frères (Lestrem, France).

Dough samples

A dough was made from 20g semolina hydrated at 53% (dry basis) with deionized water and mixed for about 2 min. In the case of supplemented dough, monohydrated maltose was added ranging from 0 to 3200 mg for each 20g semolina. The dough was sheeted in a De Lellis sheeter (Genova, Italy; rolls diameter: 5 cm; rotation speed: 50 rpm; roll gap: 0.40 mm). Between two sheetings, the strip of dough was folded 3 times and turned a quarter before being inserted again between the rolls. Discs were then cut in each dough piece with a punch (diameter: 2 cm) and sealed in plastic bags under partial vacuum. Samples were heated by immersion in a water bath at 65 or 70°C for various times, whereas control samples were immediately frozen at −20°C. After heat treatment each bag was rapidly cooled in thawing ice, then frozen at −20°C. All samples were freeze-dried and ground in a laboratory mill. Particle size after milling was below 500 µm.

Solubilization of β-amylase

β-amylase was extracted by suspending either 1g semolina or freeze-dried ground dough in 10 mL water at 4°C for 1 hr under continuous stirring and then centrifuged (10 min, 12000 × *g*).

Electrophoresis

SDS-PAGE was carried out according to Payne and Corfield (1979) as modified by Autran and Berrier (1984).

β-Amylase activity

The activity of β-amylase in semolina and dough pieces water extracts was measured by the Betamyl method (MegaZyme, Sydney, Australia) with *p*-nitrophenyl-α-D-maltopentaose (PNPG5). Results were given in µmoles of PNPG5 hydrolysed.min^{-1}.g^{-1} of semolina or dough (Mathewson and Seabourn, 1983; Mc Cleary and Codd, 1989).

Determination of maltose, sucrose and glucose

Extraction of sugars was carried out according to Henry (1985) with a few modifications. Semolina or ground dough (125 mg) was extracted twice with 2 mL ethanol 80% (v/v) for 10 min in a water bath (85°C) with intermittent stirring. The pellet was then washed twice with 2 mL ethanol 80% (v/v) for 5 min at room temperature under continuous stirring. The four supernatants were pooled and dried. Maltose and sucrose were measured by the "Test-Combination sucrose/D-glucose" (Boehringer, Mannheim, Germany), and glucose was measured by the glucose-oxidase method described by Colonna et al. (1981).

Kinetics of heat denaturation of β-amylase

The water extracts, whether or not supplemented with various chemicals, were divided into 1 mL aliquots into 2 mL Eppendorf® microtubes, immersed in a water bath (heated at 50, 56, 58, 60, 63 and 65°C) over periods ranging from 4 to 45 min, cooled in ice immediately after treatment and then centrifuged (10 min, at 12800 × *g*) to clarify the extract. During heat treatment, control samples were kept in ice and then centrifuged like treated samples. The β-amylase activity of the supernatants was measured by the Betamyl method. Heat denaturation of β-amylase was tested at 65°C over periods ranging from 2 to 10 min in the presence of the following additives: (NaCl) 0.5, 1 and 2M; (bovine serum albumin) 1 and 10 mg/mL; (industrial powder gluten) 10 mg/mL; (monohydrated maltose) from 2.77 10^{-3}M to 0.277M; (soluble starch according to Zulkowsky) 300 mg/mL; (soluble starch + gluten mixture) 300 mg/mL + 10 mg/mL; (sucrose) 0.277M and 2M; (monohydrated lactose) 0.138M; (polyethylene glycol 300) 7.5% (v/v); (polyethylene glycol 20,000) 10 mg/mL; (ethylene glycol) 10% (v/v); (glycerol) 50% (v/v).

RESULTS & DISCUSSION

Heat denaturation of β-amylase in water or in dough

Heat denaturation of β-amylase, extracted with water, was determined for temperatures between 50°C and 65°C and for incubations ranging from 0 to 45 min. Remaining activity was measured. Typical results at 65°C are presented (Fig 1). After a latent phase of ≈2 min, the kinetics of denaturation were first order, the semi logarithmic representation of the activity left as a function of time was linear (▲, Fig. 1). The lag phase was due to the rise in temperature inside the Eppendorf® tube. After 2 min incubation, the water bath had reached 92.5% of the target temperature, and 2 min more were necessary to reach 99.5% of the target temperature. Those values were obtained from six different experiments carried out at water bath temperatures from 55°C to 80°C. The temperature inside the Eppendorf® tube was reported using a thermo-couple probe (K type) with a 0.5 mm diameter tip (data not shown). After 4 min incubation, the temperature was steady, but remained below the water bath temperature, probably due to heat exchange between the ambiant atmosphere and the tube surface. With closed Eppendorf® tubes, such as those used for studying β-amylase denaturation, water bath and tube temperature could be expected to be closer after 4 min incubation. For each temperature studied $t_{1/2}$ (time when 50% of initial activity was left) was determined by the kinetics of heat denaturation, for incubation times exceeding 4 min (Fig. 2). At 50°C, β-amylase is not inactivated; this supported the findings of Meyer et al. (1953) who report a loss of activity only above 50°C. Between 60 and 65°C, $t_{1/2}$ decreased by a factor of about 5. shifting from 13.6 min to 2.4 min.

Dough samples, sheeted only once, were treated at 65°C over periods of 0 to 8 min. The β-amylase activity was measured in the water extract of dough samples. Under those conditions, the kinetics of denaturation were also first order (●, Fig. 1). The rate of denaturation of β-amylase was similar to that previously reported in heated water extracts. However, no latent phase was observed. In dough, the required temperature (65°C) was reached in <10 s at the core of dough samples 0.4 mm thick (with a thermo-couple probe enclosed in a dough piece).

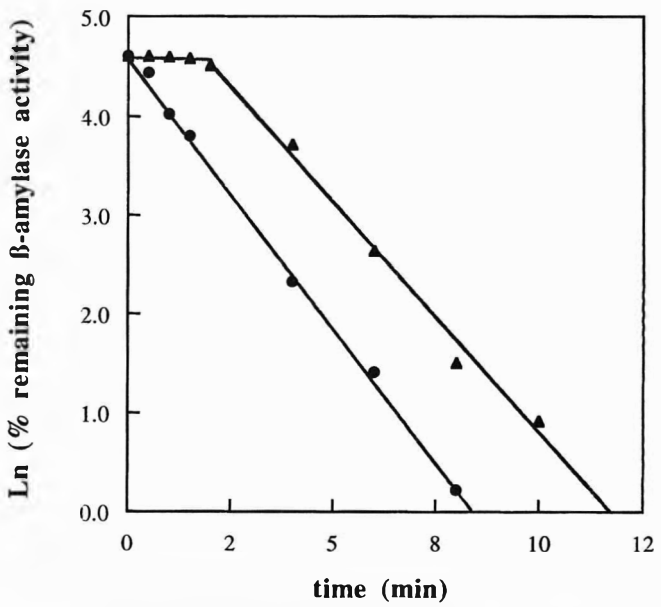


Fig. 1—Kinetics of heat denaturation at 65°C of β-amylase in water (▲) or in dough pieces (●). Ln: neperian logarithm of the percentage of remaining β-amylolytic activity.

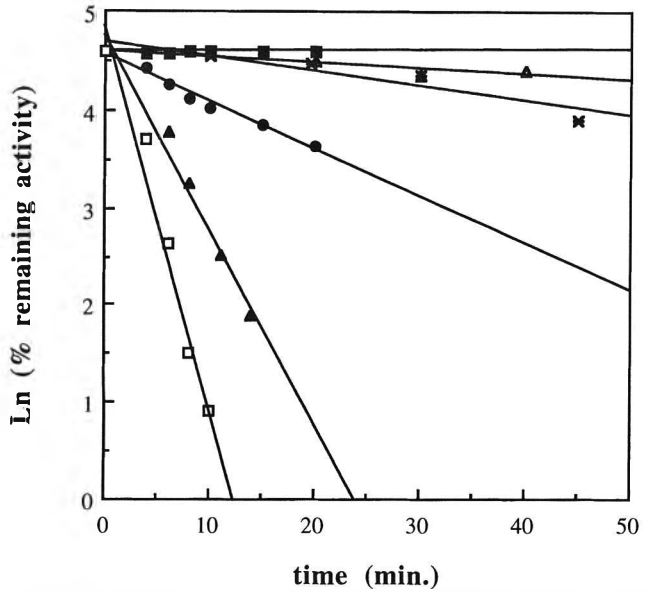


Fig. 2—Kinetics of heat denaturation of β-amylase in water. Ln: neperian logarithm of the percentage of remaining β-amylolytic activity. Heat denaturation temperatures were: 50°C (■), 56°C (△), 58°C (*), 60°C (●), 63°C (▲), 65°C (□). The velocity constants of denaturation were respectively: $-7.55\ 10^{-4}$ min^{-1}, $5.79\ 10^{-3}$ min^{-1}, $1.5\ 10^{-2}$ min^{-1}, $4.83\ 10^{-2}$ min^{-1}, $19.76\ 10^{-2}$ min^{-1} and $38.9\ 10^{-2}$ min^{-1}.

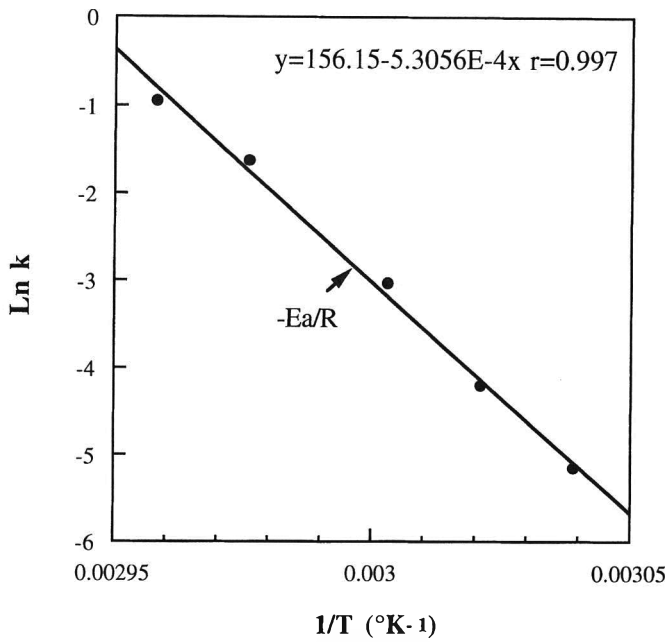


Fig. 3—Energy of activation (E_a) of heat denaturtion of β-amylase in water. (T): temperatures ranging between 56°C and 65°C are indicated in Kelvin^{-1}.

Activation energy (E_a) for the heat denaturation of β-amylase

The above results allowed us to determine graphically an activation energy (E_a) of 439 kJ/ moles corresponding to the heat denaturation reaction of β-amylase in solution heated between 56°C and 65°C (Fig. 3). Ea was computed according to the Arrhenius law: Lnk = -Ea/R(1/T)+c with k = constant of observed velocity, R = universal gas constant, T = temperature in °K and c = constant. The values of E_a connected with heat protein denaturation were higher than those of other chemical reactions. For trypsin, ovalbumin and peroxidase, heat denaturation E_a values of 167, 552 and 773 kJ/mole were respectively reported by Cheftel et al. (1985). The activation energy of the β-amylolysis between 20 and 50°C was only 39 kJ/mole (Meyer et al., 1953).

Effect of chemical factors on heat denaturation in aqueous solution at 65°C

Enzymes are known to be stabilized by addition of various compounds like polyhydric alcohols such as glycerol or sucrose as well as salts or polymers (Schmid, 1979). Tests were made to evaluate effects of NaCl, of sugars like lactose and sucrose (maltose analogues) and of polymers or polyols (PEG, ethylene glycol and glycerol). In the same way, it is generally held that ligand binding cause either stabilization or labilization of enzymes or no effects. Therefore, the effects of starch and maltose, respectively substrate and product of the β-amylolisis, were investigated. The effect of polymers like proteins was also tested (bovine serum albumin, gluten proteins). A stability factor α (ratio $t_{1/2}$ with chemical factor on $t_{1/2}$ control) (Larreta Garde, 1990) was determined for assessing the influence of chemical factors on denaturation (Fig. 4A). We also assessed the initial activity rate β (ratio of β-amylase activity after 1 hr incubation either with or without chemical factors). The latter varied little from one chemical factor to the other (Fig. 4B). None of the substances tested had notable influence on the stability of the enzyme, except for glycerol 50% (β = 1.2).

Effect of ionic strength

The effect of ionic strength on velocity of heat inactivation of β-amylase was measured by varying the molar concentration of a neutral salt (NaCl) from 0.5 to 2M at 65°C (Fig. 4A). NaCl facilitated heat denaturation as shown by $t_{1/2}$, which confirmed the findings of Lorient and Mesnier (1990). Proteins are relatively insoluble in pure water and interact through their ionic sites. Addition of salt improves their solubility while increasing their tendency to stretching and lowering their resistance to heat.

Effect of bovine albumin and gluten

The effects of two concentrations of albumin (1 and 10 mg/mL) as well as the influence of wheat storage proteins, added in the form of industrial gluten, were determined. The equivalent of the gluten content of 1g semolina, was added to the aqueous solution (10 mg/mL extract). Gluten is poorly water soluble so a suspension was obtained. The effect on the stability factor was slightly negative (Fig. 4A), indicating possible interactions between β-amylase and these proteins. Gluten addition made the resulting compound slightly more susceptible to heat denaturation.

Effect of soluble starch and a soluble starch/gluten mixture

Enzymes are more stable to heat in the presence of either their substrate or substrate analogues (Moriyama et al., 1977; Greenberg et al., 1985; Ward and Moo-Young, 1988). With 30% soluble starch a highly negative effect on the stability of β-amylase was observed (Fig. 4A). In such conditions, the stability factor was reduced to 0.4. The soluble starch concentration used widely exceeded the K_m of the complex β-amylase/soluble starch (0.12% for soluble starch, personal results), thus the enzyme was saturated with substrate. We concluded that, when β-amylase is complexed with soluble starch, it becomes more susceptible to heat denaturation than the free enzyme. These results were different from those reported by Ward and Moo-Young (1988) for α-amylase, that high substrate concentrations (30 to 40%) had a positive effect on thermostability of this enzyme. This could be explained by the theory of Silverstein and Grisolia (1972). According to them, if substrate leads to a conformation of higher internal energy, denaturation through the

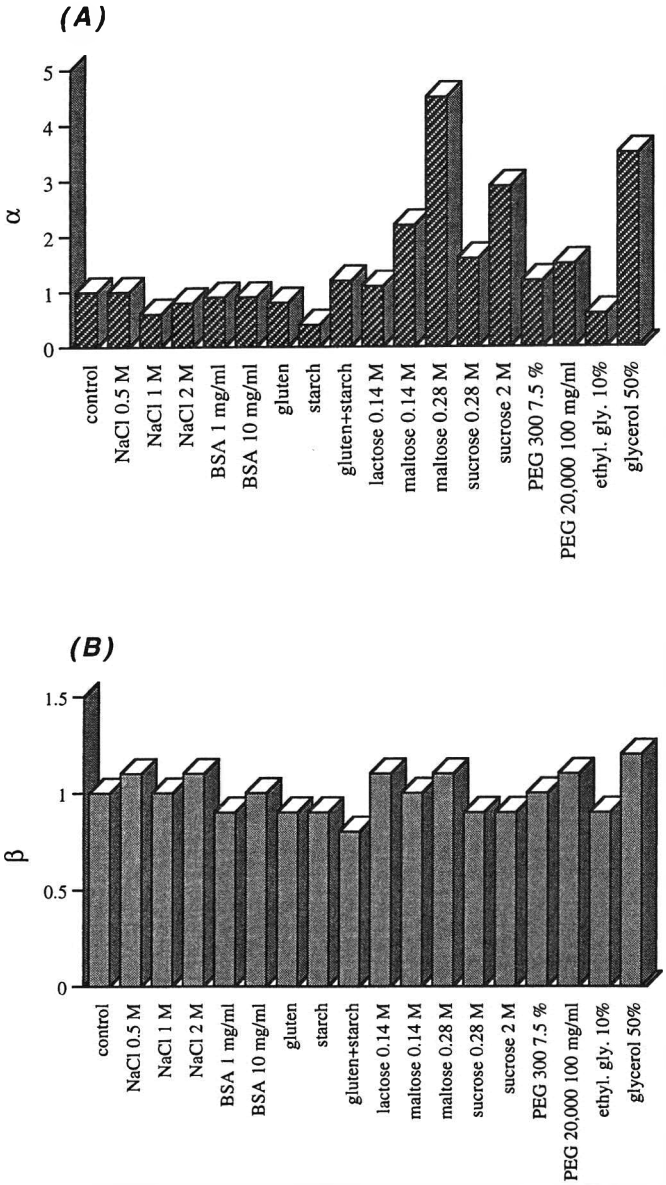


Fig. 4—(A) Stability factor (α ▨), (B) initial activity (β ▒) of β-amylase in water in the presence of various chemical factors. Heat denaturation of β-amylase was tested in the presence of chemical factors after variable periods of incubation at 65°C. (α) ratio of the $t_{1/2}$ with or without chemical factors. (β) ratio of the activities with or without chemical factors and without heat treatment.

critically activated state D* is facilitated. Conversely if by substrate binding a conformation of lower internal energy results, the complex is better protected against denaturation.

The combination soluble starch/gluten, had a positive effect on stability. Therefore, the presence of gluten counterbalanced the negative effect of starch. No explanation has been found for this result, though a tertiary complex may conceivably have formed : starch/enzyme/gluten, about as resistant to heat as the original enzyme.

Effects of maltose and disaccharide analogues

No increase in thermostability was observed in the presence of a high concentration of substrate. Thus, we tested the effects of the product of β-amylolysis (maltose) and that of disaccharide analogues (sucrose and lactose) at equivalent molar concentrations (Fig. 4A). Because of its low water solubility, lactose was used at molarity of 0.138M (47 mg/mL).

For the same molar weight of maltose, the stability factor (α) obtained was 2.2 whereas almost no protective effect occurred in the presence of lactose. The enzyme became more stable, the higher the concentration of maltose and sucrose. At equivalent molar concentrations however, maltose protected β-amylase better than sucrose. Maltose certainly acts via a direct interaction with the enzyme since it is generally known to work as an inhibitor of β-amylolysis (Thoma et al., 1971). The protective effects of analogues such as sucrose and lactose were probably due to decreasing the amount of free water surrounding the enzyme.

These findings supported those of several reports, such as Greenberg et al. (1985) who related that thermostability of lactase was multiplied by 7 in the presence of its substrate (lactose). This became 2 and 1.5 respectively, in the presence of glucose and galactose (products of the reaction) whereas maltose (lactose analogue) had no effect. Banks and Greenwood (1968) also reported the positive effects of maltose on the stability of sweet potato β-amylase in very diluted solution.

Note that sugars improve thermostability of enzymes. Gekko and Koga (1983) reported a stabilizing effect of sugars on heat denaturation of collagen, correlated with −OH groupings in equatorial position. Consequently, the effect of lactose is intermediate, between those of maltose and sucrose with 6.67 −OH/ molecules in equatorial position vs 7.62 for maltose and 6.5 for sucrose.

Effect of polyols

Many researchers (see Larreta Garde review, 1990) described the stabilization of enzymes by sugars and polyols. These compounds generally do not interact much with enzymes whereas they have many interactions with water favoring a high degree of water organization around proteins and reducing the disorder caused by temperature rise (Back et al., 1979; Lee and Timasheff, 1981; Gekko and Koga, 1983; Monsan and Combes, 1984). We tested the effects of two polyethylene glycols, ethylene glycol, and glycerol (Fig. 4A). The protective effect of glycerol was unquestionable (stability factor 3.5), though, despite the high concentration (50%), it was not as effective as a lower concentration of maltose (100 mg/mL). Glycerol and ethylene glycol act mainly as depressors of water activity, however ethylene glycol has an inhibiting effect ($\beta < 1$). Our results substantiate the findings of Monsan and Combes (1984) who reported that because of its short carbon chain (2 carbon atoms), ethylene glycol would interact with the internal structure of invertase. In the case of both polyethylene glycols, we observed a slight protective effect increasing as a function of molecular weight.

Importance of maltose in heat inactivation of β-amylase

Results suggested that the higher the concentration of maltose, the better the enzyme was protected. This was supported by analysis of the kinetics of heat denaturation of β-amylase in aqueous medium in the presence of increasing concentrations of monohydrated maltose, ranging from 0 to 100 mg/mL (Fig. 5).

In the presence of maltose, the kinetics of denaturation of β-amylase were always first order, but the observed constant of inactivation decreased as a function of maltose concentration. The inactivation tended toward zero in the presence of 100 mg/mL maltose. These findings indicated the probable occurrence of 2 enzymatic forms, a free one susceptible to heat denaturation and another, bound to maltose, more resistant to heat. In the presence of maltose, both forms could coexist and their equilibrium would depend on the concentration of maltose, according to the mass action law. This can be illustrated by the reaction model presented (Fig. 6) where E represents native β-amylase and D the denatured, inactivated and insoluble form. Denaturation requires a form D* in equilibrium with the native form E. In the presence of maltose L, this equilibrium is changed into a

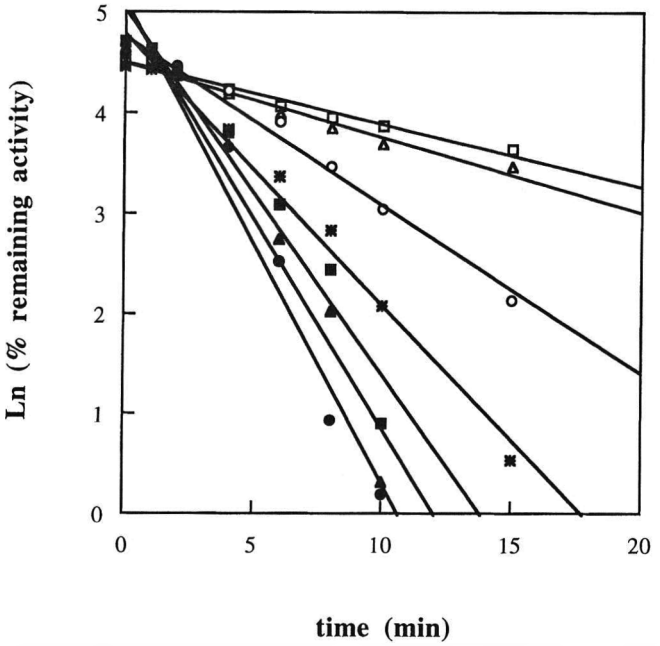
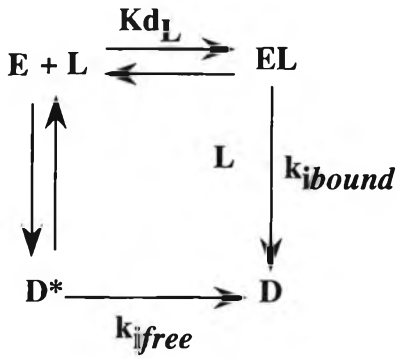


Fig. 5—Kinetics of heat inactivation of β-amylase in water as a function of maltose concentration. Concentrations of maltose: (●) 0 mg/ml; (▲) 1 mg/ml; (■) 10 mg/ml; (*) 25 mg/ml; (○) 50 mg/ml; (△) 75 mg/ml; (□) 100 mg/mL.



$$(1)\quad k_{ap} = \frac{(k_{ifree} \times Kd_L) + (k_{ibound} \times L)}{Kd_L + L}$$

$$(2)\quad \frac{1}{k_{ap}} = \frac{L}{k_{ifree} \times Kd_L} + \frac{1}{k_{ifree}}$$

Fig. 6—Heat denaturation mechanism of ;β-amylase in the presence of maltose. E: native enzyme (β-amylase); D: denatured enzyme; D*: denaturated reversible intermediate; L: ligand (maltose): ki_{free}: constant of velocity of denaturation of the free enzyme; k_{ap}: constant of observed velocity of denaturation; Kd_L: constant of dissociation of the complex β-amylase/maltose; ki_{bound}: constant of inactivation of the complex β-amylase/maltose.

compound β-amylase/maltose. The constant of observed velocity of inactivation (k_{ap}) depends thus on the dissociation constant (Kd_L) of the enzyme/maltose complex and on the denaturation velocity of both the free enzyme (ki_{free}) and bound enzyme (ki_{bound}) as shown by equation (1). If the enzyme under its maltose bound form does not undergo denaturation when it is incubated at 65°C, (i.e. ki_{bound} is null) then the expression of k_{ap} as a function of concentration of maltose (L) is hyperbolic. Equation (2) (Fig. 6) allows to linearize this relationship. The experimental

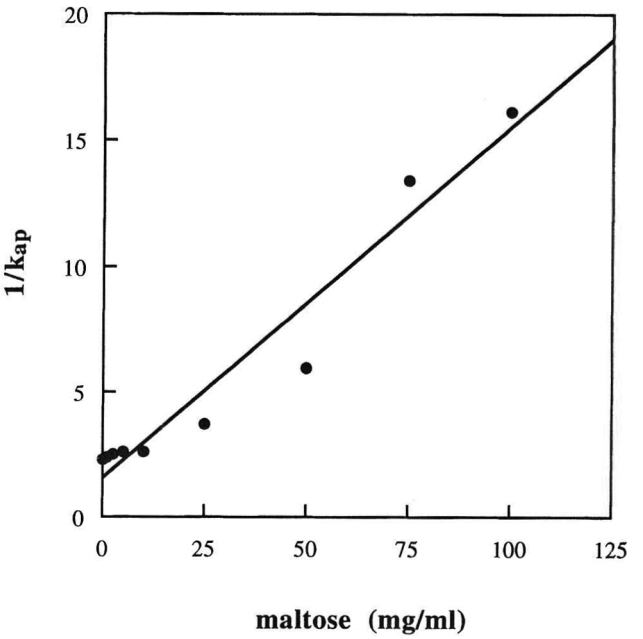


Fig. 7—Effect of maltose on the constant of observed velocity of heat denaturation of β-amylase in water. k_{ap}: constant of observed velocity of denaturation.

results (Fig. 7) confirmed our hypothesis. When $1/k_{ap}$ was represented as a function of the concentration of maltose (L) a straight line was obtained with a regression coefficient 0.945. Taking into account the different values of K_{ap} for the various concentrations of maltose (L) used, a value of 11 mg/mL maltose was computed for the constant of dissociation of the complex β-amylase/maltose (Kd_L), Ki_{free} being 0.64 min^{-1}. The protective effect of maltose on β-amylase was clearly established, moreover, both were directly linked because maltose was the product of β-amylolysis of starch.

Sucrose and maltose levels of durum wheat and durum wheat pasta

Many studies have reported the presence of maltose, glucose and sucrose in durum wheat semolina and bread wheat flours (Theander and Westerlund, 1973; Lintas and D'Appolonia, 1973; MacArthur and D'Appolonia, 1979; Kruger and Matsuo, 1982). Furthermore, fine semolina has an important level of damaged starch, potential substrate for β-amylase. The extrusion of durum wheat pasta could favor β-amylolysis which increases maltose contents of finished products. We therefore determined the levels of maltose and sucrose of semolina as well as of various dough pieces sheeted or extruded in different ways (Fig. 8). Sucrose contents were not influenced by mechanical treatment and were in agreement with published values (Kruger and Matsuo, 1982), whereas maltose levels of cv. Ardente semolina were higher than reported values (Theander and Westerlund, 1973; Lintas and D'Appolonia, 1973; MacArthur and D'Appolonia, 1979; Kruger and Matsuo, 1982). We observed a progressive increase of these levels in relation to mechanical work, i.e. positively correlated ($r = 0.999$; $p < 0.001$) to the number of sheetings imparted to dough pieces. The maltose level of spaghetti (25.4 mg/g dry basis) corresponded to that of a dough piece sheeted 20 times, this result being slightly less than reported values (Kruger and Matsuo, 1982; Lintas and D'Appolonia, 1973). According to those studies, maltose was produced during pasta processing in relation to the great increase in damaged starch due to mechanical damage during mixing, extrusion, or drying. At the initial stages of spaghetti drying, moisture and temperature are ideal for enzymatic activity, with more maltose produced, this increase being masked by the simultaneous conversion of maltose into glucose. However, in our

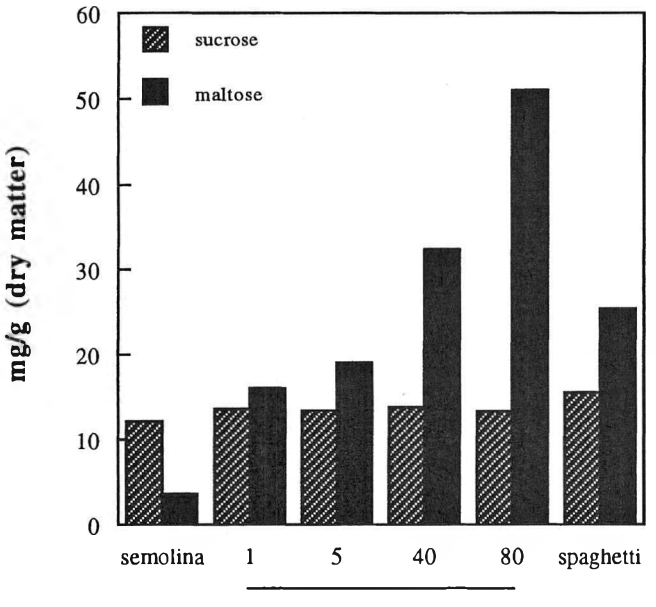


Fig. 8—Evolution of sucrose and maltose contents as related to number of sheetings.

Table 1—Contents of maltose and activity of β-amylase in heated dough pieces as related to the number of sheetings

Number of sheetings	Treatment at 70°C duration (min)	Maltose (mg/g)	β-amylase activity (μmoles PNPG5 hydrolyzed.min^{-1} .g^{-1} dough
1	0	14.3	1400
1	1.5	17.9	4
1	2.5	16.0	2
5	0	18.7	1306
5	1.5	19.4	9
5	2.5	19.5	2
40	0	29.3	1187
40	1.5	34.4	238
40	2.5	33.5	61
80	0	49.3	1144
80	1.5	47.5	585
80	2.5	56.6	517

experimental conditions maltose production only occurred during mixing and sheeting. A dough piece was sheeted 60 times and separated into 2 pieces: one was frozen immediately while the second was kept at 20°C, for 6 hr, in an hermetically sealed plastic bag. Both dough pieces were freeze-dried and crushed, then their maltose levels were measured. The content of the instantly frozen dough piece was 29 ± 2.7 mg/g whereas that of the rested dough was 31.8 ± 1.2 mg/g. Maltose development is therefore limited in dough at rest, which substantiated the results obtained for bread dough by Potus et al. (1994).

In dough, amylolysis probably reaches its limits quickly. The limitation observed was explained by Drapron and Guilbot (1962a, 1962b). They assumed that a topochemical reaction occurs at contact points between enzyme and substrate. The rigidity of the medium causes an increase in concentration of reaction products which may lead to local decreases in water activity. Whereas mixing or mechanical work in general, facilitates molecular interactions by multiplying contacts between enzyme and substrate and causing production of more maltose (Potus et al., 1994).

Heat denaturation of β-amylase in durum wheat dough pieces

Water-soluble proteins extracted from dough pieces sheeted 1 to 80 times whether or not heated at 65°C for 4 min were analyzed by SDS-PAGE (Fig. 9). After 4 min heat treatment at 65°C, the band corresponding to β-amylase (arrow, Fig. 9) became fainter the fewer the sheetings imparted to the dough piece, indicating a loss of solubility in water. Resistance to heat was thus increased by the number of sheetings imparted to the dough. The protective effect of increasing proportions of maltose, described in water medium was confirmed in dough.

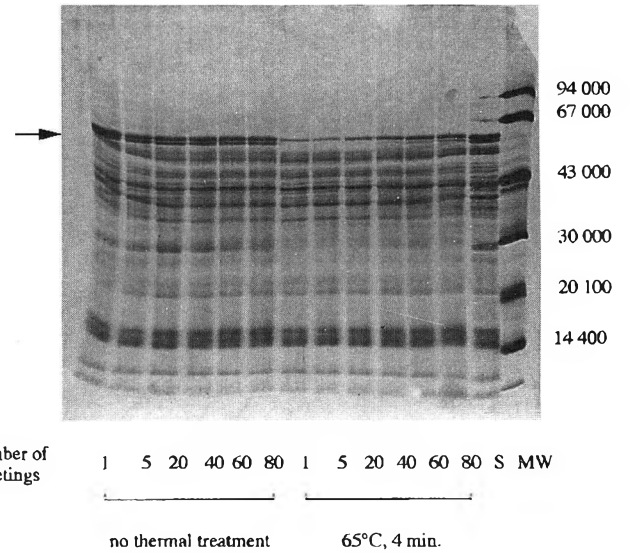


Fig. 9—SDS-PAGE of water-soluble proteins of sheeted dough pieces whether heated or not at 65°C for 4 min. (↘): β-amylase; S: semolina; MW: molecular weight markers.

Correlation between maltose levels of sheeted dough pieces and their β-amylase activity

The activity of β-amylase contained in sheeted dough pieces whether or not treated at 70°C was measured by the Betamyl method (Table 1). In non-treated samples, the activity of β-amylase decreased slightly in relation to the number of sheetings ($r = -0.915$) and to the level of maltose ($r = -0.903$) which depends on the number of sheetings. Dough samples heated for 1.5 or 2.5 min showed a significant positive correlation between the concentration of maltose and the activity of β-amylase ($r = 0.988$ with $p < 0.01$ for dough pieces treated at 70°C for 1.5 min and $r = 0.951$ with $p < 0.05$ for those treated at 70°C for 2.5 min). Thus maltose produced during sheeting somehow protected β-amylase against heat inactivtion.

Control dough pieces

To verify the protective effect of maltose produced during mixing and sheeting, several dough pieces were made from semolina and variable proportions of maltose duplicating the effects of sheeting (see maltose levels obtained in Fig. 8). Dough pieces were sheeted only once for flattening the dough evenly and repeatably in view of heat treatment. Water-soluble proteins extracted from these dough pieces were analyzed by SDS-PAGE (Fig. 10). The staining of β-amylase band was almost constant in non-treated samples (arrow, Fig. 9) whereas in the case of heated samples, the enzyme was obviously protected by high concentrations of maltose. The activity of β-amylase in the same extracts was measured by Betamyl method (Table 2). The expected protective effects of maltose can be observed. Activity of β-amylase in dough pieces supplemented with maltose or sheeted and treated at 65°C were compared (Fig. 11) as a function of their levels of maltose (added or produced by sheeting). The activity increased proportionally to the contents of maltose, whether it came from supplementation or from catalytic activity of β-amylase (sheeted dough pieces). In the case of supple-

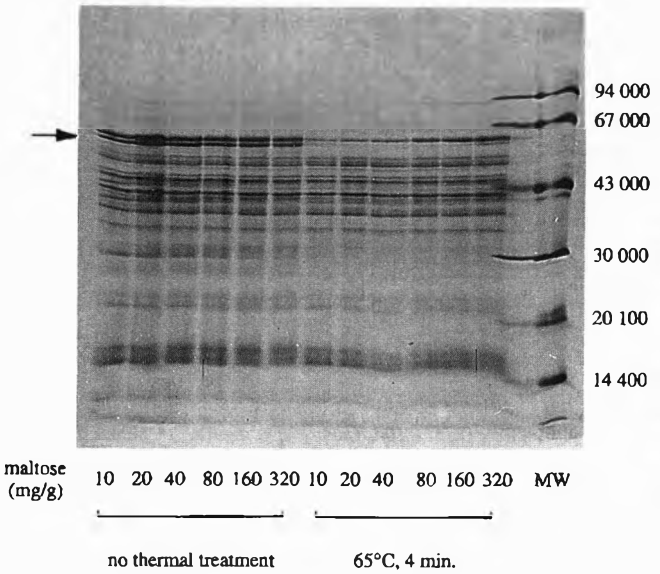


Fig. 10—SDS-PAGE of water-soluble proteins of dough pieces supplemented with maltose, sheeted only once and heated at 65°C for 4 min. (↘): β-amylase; MW: molecular weight markers.

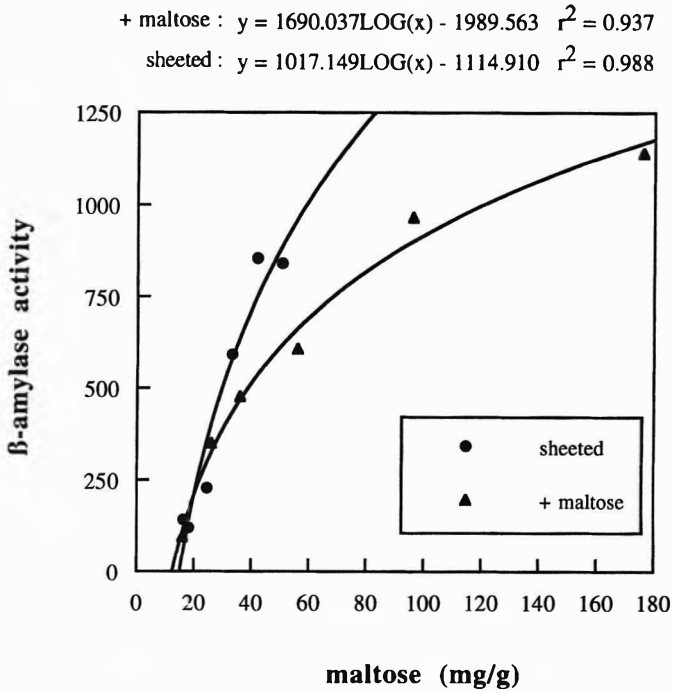


Fig. 11—Activity of β-amylase in dough pieces, either supplemented with maltose or sheeted then heated at 65°C for 4 min, as related to estimated or determined levels of maltose (mg/g). Activity expressed in hydrolyzed μmoles of PNPG5.min^{-1}. g^{-1} of freeze-dried dough.

Table 2—Effects of number of sheetings on activity of β-amylase in dough pieces supplemented with maltose and heated at 65°C (a) or not (b)[a]

(a) Doughs: 65°C (4 min.)			(b) Control doughs		
Number of sheetings	Added maltose (mg/g semolina)	β-amylase activity	Number of sheetings	Added maltose (mg/g semolina)	β-amylase activity
1	0	141	1	0	1500
5	0	119	5	0	1420
20	0	228	20	0	1350
40	0	592	40	0	1269
60	0	855	60	0	1164
80	0	841	80	0	1051
1	0	94	1	0	1550
1	10	349	1	10	1649
1	20	477	1	20	1618
1	40	607	1	40	1694
1	80	967	1	80	1555
1	160	1141	1	160	1461

[a] The activity of β-amylase is expressed in μmoles of hydrolysed PNPG5. min^{-1}. g^{-1}.

mented dough pieces however, the protective effect of maltose proved two times less efficient. If the constant of dissociation of the complex enzyme/maltose is not dependent on the environment of the enzyme (water or dough) then the occurrence of local overconcentration would explain that β-amylase was better protected in sheeted dough pieces. Consequently, the contents of maltose measured in sheeted dough pieces may be average levels.

CONCLUSION

β-AMYLASE can withstand high temperatures in the presence of chemical factors such as sugars and polyols that also protect other enzymes. In water extracts, β-amylase was very well protected by maltose and also, to a lesser extent, by sucrose and glycerol. Both substances likely stabilize the enzyme by altering the degree of organization of water. In durum wheat dough pieces, the content of maltose was increased by mixing and sheeting and the higher the level of maltose, the more enzymatic activity was retained after heating. Semolina hydration and mixing allow hydrolysis to start. β-Amylase reacts with starch damaged during milling but, the small amount of damaged starch, the short contact time and the low temperature retard hydrolysis. Amalgamation i.e. dough formation takes place during extrusion, allowing continuous renewed contact between enzyme, substrate and product, thus limiting inhibition of β-amylase by maltose. Shearing forces produce damaged starch allowing level of substrate to rise. After extrusion of dough, enzymatic activity is quickly stopped. β-Amylolysis benefits from an increased input of mechanical energy and can be maintained during rising temperatures. Excessive extrusion speed is likely to cause an increase in maltose level which could impair cooking quality and color of dried pasta. The proportion of maltose produced during pasta processing could be a valuable indicator of efficiency of mixing and probably marks shear strain as well.

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Ms received 8/15/94; revised 5/3/95; accepted 5/7/95.

We gratefully acknowledge J. Abecassis, J.C. Autran, and P. Feillet for valuable advice and comments.

Textural Characteristics of Wholewheat Pasta and Pasta Containing Non-Starch Polysaccharides

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ABSTRACT

Pasta enriched with nonstarch polysaccharides, and wholewheat pasta were assessed for cooking quality. Xanthan gum improved pasta firmness when added at 1 and 2%, without affecting moisture uptake or degree of swelling when cooked for a constant time. Dynamic rheological testing indicated development of a network structure with addition of gums which contributed to overall pasta firmness. Food grade pea fiber, at 5 and 10%, caused moderate reduction in firmness. Wholewheat pasta was similar in firmness to pasta with 10% pea fiber, as measured by Instron. Dynamic rheometry measurements indicated a weak network in wholewheat pasta. Small strain dynamic tests were more sensitive to subtle changes in network structure than were large deformation compression tests.

Key Words: wholewheat pasta, nonstarch polysaccharides, texture, firmness, cooking quality

INTRODUCTION

SEVERAL PASTA PRODUCTS are available containing nonstarch polysaccharides. Canned or frozen pasta products may contain various gums to enhance texture, or act as freeze-thaw stabilizers (Teague, 1988). Several such products are also high in dietary fiber, including wholewheat pasta and pasta containing refined pea fiber.

Little information is available on the use and function of gums in pasta products. Published studies have primarily focused on gums as bulking agents to produce a low glycemic response in diabetics (Gatti et al., 1984; Briani et al., 1987; Carra et al., 1990). Interest has increased in the use of gums as a source of soluble dietary fiber (Andon, 1987; Ink and Hurt, 1987; Theander et al., 1993). Addition of small amounts of gum to a formulation can make considerable contribution to product fiber content (Andon, 1987). Some data are available on the effects of various gums on wheat starch gelatinization (Christianson et al., 1981), wheat tortilla quality (Friend et al., 1993), and other cereal based products (Ferrero et al., 1993, Miller and Hoseney, 1993, Ward and Andon 1993). Effects of water-soluble gums on pasta cooking quality, however, have not been reported.

Similarly, little information is available on cooking characteristics of pasta with high levels of insoluble dietary fiber. Oat fiber has been studied as a dietary supplement in pasta products (Dougherty et al., 1988), but was not rated highly because of difficulty in extrusion and barely acceptable texture ratings in the final product. Some work has been reported on pasta enriched with durum bran (Kordonowy and Youngs, 1985) and that produced from high extraction rate milling of common wheat (Sahlstrom et al 1993). Kordonowy and Youngs (1985) found a decline in pasta cooking quality as bran content was increased to a maximum of 30% in the final product. Sensory panelists that were regular consumers of wholewheat bread preferred pasta supplemented with 10% bran over higher levels of bran. Pasta with no added bran was rated highest overall for flavor, texture and color. Sahlstrom and coworkers (1993) produced wholewheat spaghetti from common wheat which displayed reduced firmness and increased stickiness when compared with commercial durum spaghetti.

Our objective was to assess textural characteristics and cooking quality of pasta enriched with various gums and pea fiber, as well as that produced from 100% extraction durum and common wheat.

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MATERIALS & METHODS

Wheat samples

Wheat samples included a composite of No. 1 Canada Western Amber Durum (CWAD) and No. 2 CWAD from the 1990 Grain Research Laboratory harvest survey, and a similar composite from the 1991 survey. A commercial sample of No. 1 Canada Western Red Spring (CWRS) was also used. The CWAD samples were of similar protein content, 12.1% and 12.5% (13.5% mb) respectively. The CWRS wheat had a protein content of 13.5% (13.5% mb).

Milling

Wheats were cleaned and tempered overnight to 16.5% moisture content. The CWAD wheats were experimentally milled to yield semolina with a degree of refinement of 0.66% and 0.64% ash, respectively, CWRS farina ash content was 0.45%, expressed on 14% moisture basis. The protein content of both semolina samples was 11.5%; the farina protein was 12.1% (14% mb).

Wholewheat samples were prepared by initially milling to granulars (semolina/farina and flour) using a modified flow on a 25-cm Ross mill (Dexter et al., 1988). All feed stocks were blended and then reduced on the Ross mill scratch rolls at the closest possible setting (≈0.03 mm) until all stocks passed through a 501 μm sieve. The granulars and reduced feed were then combined and blended.

Nonstarch polysaccharides

Water-soluble arabinoxylans were isolated from CWAD1 semolina according to the method of Izydorczyk et al. (1990) and added back to produce pasta with 1% and 2% enrichment (flour weight basis). Gum arabic, guar gum, locust bean gum and xanthan gum, in agglomerated form for easier hydration, were supplied by Zumbro/IFP, Inc. (Hayfield, MN) and were also used at 1% and 2%. Levels in excess of 2% presented difficulties in extrusion.

Pea fiber, used commercially for production of high fiber pasta, was the generous gift of Woodstone Foods Corp. (Winnipeg, MB, Canada) and was added at levels of 5% and 10%, within the range recommended by the supplier. Pea fiber was described by the supplier as ≈80% insoluble and 20% soluble dietary fiber.

Pasta processing

The micro spaghetti-making process of Matsuo et al. (1972) was used for preparation of samples for initial screening of the effects of various polysaccharides on pasta. A single bulk semolina sample was used as the base for all fortified samples. The same durum wheat was milled to produce a wholewheat semolina.

Samples which were further studied and tested by dynamic rheometry were processed using a Demaco semicommercial laboratory press (De Franscisi Machine Co., Brooklyn, NY) under conditions described by Matsuo et al. (1978). A die with 0.8 × 20 mm apertures (Maldari and Sons, Brooklyn, NY) was used to produce flat noodles. The water absorption levels (normally 26% on a 14% mb) of pasta containing added

Table 1—Quality of optimum cooked noodles

Sample	Instron		Bohlin	
	Peak force (kg)	Peak energy (kg-mm)	G' (kPa)	η' (Pa·s)
CWAD 1				
CWAD-A[e]	1.075[d]	0.350[c]	4.20[b]	234[b]
1% LBG[f]	1.118[cd]	0.390[c]	5.06[ab]	361[ab]
2% LBG[f]	1.235[bc]	0.453[b]	6.27[ab]	447[a]
1% Xanthan	1.326[b]	0.409[bc]	5.27[ab]	331[ab]
2% Xanthan	1.650[a]	0.507[a]	6.63[a]	442[a]
CWAD-B[e]	1.237[a]	0.426[a]	5.88[b]	356[b]
Wholewheat	0.875[b]	0.274[b]	8.54[a]	670[a]
5% Pea Fiber	0.926[b]	0.329[b]	5.72[b]	366[b]
10% Pea Fiber	0.894[b]	0.304[b]	5.57[b]	378[b]
CWAD 2				
CWAD2[e]	1.193[b]	0.427[b]	5.10[b]	290[b]
2% Xanthan	1.589[a]	0.559[a]	5.61[b]	381[b]
5% Pea Fiber	0.955[c]	0.339[c]	4.86[b]	257[b]
Wholewheat	0.957[c]	0.330[c]	10.21[a]	727[a]
CWRS				
CWRS[e]	1.173[b]	0.396[ab]	5.16[b]	332[b]
2% Xanthan	1.433[a]	0.469[a]	5.09[b]	324[b]
5% Pea Fiber	0.980[c]	0.332[b]	4.90[b]	318[b]
Wholewheat	0.947[c]	0.302[b]	11.37[a]	808[a]

[a-d] Values within a group followed by different letters are significantly different, LSD $\alpha = 0.05$.

[e] Control samples processed containing no additives with their respective groups.

[f] LBG = locust bean gum.

non-starch polysaccharides, as well as the wholewheat pasta were increased in order to achieve the best dough viscosity for extrusion. Gum supplemented doughs were extruded at 30% absorption (14% mb). Pea fiber supplemented pasta and wholewheat pasta both required an increase to 32% absorption (14% mb). All samples were dried using the 39°C drying cycle described by Dexter et al. (1981).

Pasta samples were segregated into test groups during processing and drying. Water-soluble polysaccharide enriched CWAD1 samples were processed as a group and the pea fiber and wholewheat CWAD1 processed together as the second group. A control sample, of unenriched semolina, was processed along with each group.

In order to corroborate results based on CWAD1 semolina, the second durum (CWAD2) and CWRS were processed with the addition of 2% xanthan gum, and 5% pea fiber, and as wholewheat to form an additional two groups. Each group included an unenriched control sample, CWAD2 semolina for group 3 and CWRS farina for group 4.

Cooked noodle weight, swelling index and moisture content

All tests were performed in duplicate at each cooking time. Noodle cooked weight, swelling index and moisture content were determined using 10-g samples (12% mb) cooked in 600 mL of boiling tap water. After cooking for the prescribed time each sample was drained and placed in cold water for 1 min to arrest further cooking. Samples were again drained and transferred to a 100 mL graduated cylinder containing 50 mL of distilled water where the displacement volume was measured. Each noodle sample was again drained and weighed, providing the cooked weight. Moisture content of cooked noodles was determined by freeze-drying the cooked, drained noodles and comparing the resulting weight of freeze-dried material with the cooked weight.

Cooking loss

Cooking losses were determined in triplicate by freeze-drying the water retained from cooking 5-g pasta samples as described by Dexter and Matsuo (1979). The iodine binding method of Matsuo et al. (1992) was used to provide an indication of the amount of amylose leached into the cooking water.

Instron firmness

Cooked noodle firmness was determined as peak force and peak energy using an Instron Universal Testing Machine (model 4201, Instron Corp., Canton, MA) equipped with a 10-kg load cell. Samples were tested in triplicate in a randomized block design. The method was a modification of AACC (1983) method 16–50. Samples were cooked to optimum time (when the central white core disappeared) and 12 min past optimum. Preparation and testing of samples were essentially as described by Edwards et al. (1993).

Dynamic rheometry

Dynamic viscoelastic properties of cooked noodles were measured in triplicate for both cooking times in a randomized complete block design using a Bohlin VOR rheometer (Bohlin Reologi, Edison, NJ). Parallel plate geometry of 15 mm was used with a torque element of 93.2 g-cm. Measurements were taken at 25°C in a 0.1 to 5.0 Hz frequency range and ≤ 1% strain. Preliminary testing indicated that at these levels of strain the requirement for linear viscoelasticity of the samples was met. Sample preparation and testing was as previously described (Edwards et al., 1993). Data at a frequency of 0.5 Hz were used for all statistical comparisons.

Statistics

Analysis of variance and regression procedures of the SAS software system, release 6.08 (SAS Institute, Cary, NC), were used for statistical analyses.

RESULTS & DISCUSSION

Preliminary studies

Micro-processed spaghetti samples were tested using the Instron to determine effects of non-starch polysaccharides on textural characteristics. Addition of durum arabinoxylan and water-soluble gums appeared to have little effect on textural quality of spaghetti, with the exception of xanthan gum at 1% and 2% (data not shown). The same trends were observed for overcooked samples. The second group of spaghetti samples tested, with increased insoluble dietary fiber content, displayed good firmness (data not shown). The 5% pea fiber enriched spaghetti was similar in firmness to the control, while 10% pea fiber and wholewheat samples were moderately reduced in firmness.

Dynamic rheometer and instron studies

We expanded the study by including dynamic rheological testing of selected samples to provide additional information on effects of nonstarch polysaccharides on pasta texture. Small strain dynamic tests are useful to probe mechanical properties of starch-based systems while preserving specimen structure, thus complimenting data obtained by large deformation techniques such as Instron testing.

Strong relationships between Instron firmness and the dynamic rheometry parameters storage modulus, G', and dynamic viscosity, η', of cooked extruded noodles have been established (Edwards et al., 1993). We decided to include xanthan gum because of its strong improvement of firmness, as well as locust bean gum, which appeared to have relatively minor influence on texture of micro-processed spaghetti. Wholewheat and pea fiber were also included as these are both commercially available products. The samples were processed into flat noodles to provide an appropriate surface for the parallel plate geometry of the rheometer. The same groupings were used as for Instron testing.

Trends observed in this second phase of CWAD1 testing concurred with results of preliminary studies using micro-processed spaghetti. At optimum cooking time differences ($P < 0.05$) occurred between Instron firmness values of both peak force and peak energy for the gums and corresponding control samples (Table 1). The 2% xanthan gum was firmer than all other samples in this group (by LSD, $\alpha = 0.05$). The 1% xanthan gum and 2% locust bean gum were intermediate in firmness, while 1% locust bean gum and control samples were not different. Similar trends were observed in overcooked samples (Table 2).

Wholewheat and both the 5% and 10% pea fiber samples of CWAD1 were less firm as measured by the Instron than the control sample both when cooked to optimum (Table 1) and

Table 2—Quality of overcooked noodles

Sample	Instron		Bohlin	
	Peak Force (kg)	Peak Energy (kg-cm)	G' (kPa)	η' (Pa-s)
CWAD1				
CWAD-A[d]	0.396[c]	0.172[c]	2.10[b]	127[b]
1% LBG[e]	0.581[ab]	0.280[ab]	2.27[b]	142[b]
2% LBG[e]	0.531[bc]	0.263[ab]	2.08[b]	139[b]
1% Xanthan	0.495[bc]	0.198[bc]	1.97[b]	127[b]
2% Xanthan	0.715[a]	0.310[a]	3.02[a]	188[a]
CWAD-B[d]	0.529[a]	0.253[a]	1.86[b]	107[b]
Wholewheat	0.431[bc]	0.177[b]	4.78[a]	259[a]
5% Pea Fiber	0.365[c]	0.162[b]	1.43[b]	82[b]
10% Pea Fiber	0.362[c]	0.158[b]	1.59[b]	89[b]
CWAD2				
CWAD2[d]	0.439[b]	0.194[b]	1.38[c]	78[c]
2% Xanthan	0.692[a]	0.280[a]	3.23[b]	181[b]
5% Pea Fiber	0.421[b]	0.185[b]	1.38[c]	81[c]
Wholewheat	0.403[b]	0.171[b]	5.62[a]	307[a]
CWRS				
CWRS[d]	0.426[b]	0.186[ab]	1.44[c]	97[c]
2% Xanthan	0.657[a]	0.237[a]	2.72[b]	175[b]
5% Pea Fiber	0.314[b]	0.129[b]	1.46[c]	98[c]
Wholewheat	0.386[b]	0.155[b]	4.87[a]	281[a]

[a-c] Values within a group followed different letters are significantly different, LSD α = 0.05.
[d] Control samples processed containing no additives with their respective groups.
[e] Locust bean gum.

Table 3—Loss to cooking water

Sample	Optimum		Overcooked	
	Cooking loss[b] %	Iodine binding[c] abs	Cooking loss[b] %	Iodine binding[c] abs
CWAD1				
CWAD-A[d]	5.6	0.646[a]	13.1[c]	1.565
1% LBG	6.2	0.596[ab]	13.2[c]	1.501
2% LBG	5.9	0.592[ab]	14.4[bc]	1.245
1% Xanthan	7.0	0.579[b]	17.6[ab]	1.450
2% Xanthan	6.5	0.488[c]	18.6[a]	1.324
CWAD-B[d]	6.4[b]	0.632[b]	12.7[b]	1.500[a]
Wholewheat	9.6[a]	0.607[b]	12.6[b]	1.142[b]
5% Pea Fiber	6.4[b]	0.780[a]	12.7[b]	1.702[a]
10% Pea Fiber	6.8[b]	0.732[a]	14.0[a]	1.517[a]
CWAD 2				
CWAD2[d]	6.8	0.676[a]	13.4[b]	1.434
2% Xanthan	7.1	0.541[b]	19.2[a]	1.357
5% Pea Fiber	7.4	0.689[a]	13.5[b]	1.518
Wholewheat	8.4	0.623[a]	13.0[b]	1.236
CWRS				
CWRS[d]	5.0[b]	0.595[ab]	14.0[b]	1.465[ab]
2% Xanthan	6.3[ab]	0.508[c]	22.2[a]	1.369[b]
5% Pea Fiber	5.7[b]	0.620[a]	13.5[b]	1.532[a]
Wholewheat	7.2[a]	0.528[bc]	10.9[b]	1.196[c]

[a] Values within a group followed by different letters are significantly different. LSD α = 0.05. Values within groups with no letters are not significantly different.
[b] Freeze-dried total dissolved solids of cooking water.
[c] Iodine binding capacity of cooking water.
[d] Control samples processed containing no additives with their respective groups.

when overcooked (Table 2). Wholewheat and pea fiber samples were not different from each other. Bran constituents in the wholewheat pasta possibly disrupted the continuity of the protein-starch matrix resulting in a weaker product as measured by Instron. Similarly, addition of pea fiber (mainly insoluble particulates) would have a disrupting effect on the protein-starch matrix of pasta, accounting for the decrease in firmness of these samples.

Trends observed in firmness of the noodles containing water-soluble gums were also apparent when analyzed by dynamic rheometry (Tables 1 and 2). The 2% xanthan gum sample was stiffer (higher G') than the control sample when cooked to optimum, indicating development of a network structure. When overcooked, 2% xanthan gum retained this network structure and was stiffer than all other samples in this group. Samples containing 1% xanthan gum and LBG were intermediate in stiffness when cooked to optimum. Both gums at 2% had higher η' in comparison with the control sample. Only 2% xanthan gum had higher η' after overcooking.

When analyzed by dynamic rheometry high fiber samples presented somewhat different results from those obtained by Instron. Samples containing pea fiber were not different from controls. Wholewheat was different from the control, as indicated by larger G' and η'.

The calculated tan δ for the gums (range between 0.20 and 0.23) and the wholewheat samples (0.25) were higher than that of the control (0.18), indicating a larger contribution of the viscous component to the structure. For the gums this was expected as that property of enhancing viscosity is often exploited in food applications of gums (Ward and Andon, 1993). The effect of increased stiffness upon addition of water-soluble gums, as evidenced by a larger G' value, may be attributable to formation of a physically cross-linked network around the starch granules trapping them during cooking. This would be more pronounced with xanthan gum which, because of its cellulosic chain backbone, has a stiff conformation and readily gives a network structure in solution (Sanderson, 1981). Starch granule swelling and filling in spaces within a well developed protein matrix may contribute to the firmness of cooked pasta (Feillet, 1984). A comparison of results for wholewheat by large and small deformation tests indicated that these samples developed a weak network structure, presumably due to the presence of endogenous cell wall polysaccharides. The network was detectable only by small strain dynamic rheometry. Under large deformation, wholewheat pasta showed similar responses to pasta fortified with pea fiber.

Correlation was good (r^2 was $0.61 \leq 0.78$) between Instron firmness parameters and those obtained by dynamic rheometry when wholewheat data were excluded from analysis, in agreement with previous results (Edwards et al., 1993).

There were no differences ($P > 0.05$) found among samples in both groups in cooked weight, degree of swelling or moisture content (data not shown). Large differences in moisture content would have an impact on dynamic mechanical properties of such systems; water acts as a plasticizer of composite materials, reducing stiffness.

Differences were not significant among gum samples at optimum cooking time when cooking losses were measured by freeze-drying of total dissolved solids. Noodles containing xanthan gum displayed higher cooking losses without corresponding increases in iodine binding capacity of cooking water when overcooked (Table 3). Some of the material leaching into the cooking water appeared to be solubilized xanthan gum. While significant, higher cooking losses did not alter the improving effect that xanthan gum had on firmness of cooked noodles.

Differences were found among samples in iodine binding capacity of cooking water at optimum cooking time. Xanthan gum samples at both levels of addition were lower in iodine binding capacity than the control, suggesting that the proportion of amylose leaching into the cooking water was lower. This supported the hypothesis that the gum forms a network around the starch granules, trapping them in place during cooking, and restricting excessive swelling and diffusion of the amylose component.

Cooking losses at optimum cooking time showed differences among samples when measured by both the freeze-drying method and iodine binding in the high fiber group (Table 3). The wholewheat pasta displayed larger cooking losses, as measured by freeze-drying, than all other samples within the group. This may be due to the presence of water soluble components found in the bran and aleurone layers. Wholewheat sample cooking water was not different in iodine binding capacity from the control when cooked to optimum. Pea fiber samples, however, had higher iodine binding levels than the control, but had similar total dissolved solids. This provides additional evidence

that pea fiber had a disrupting effect on the protein matrix, allowing more starch granules to rupture during cooking and releasing a larger proportion of amylose to the cooking water. Control tests on the iodine binding properties of pea fiber indicated that it did not react with iodine solution.

Total dissolved solids of control, wholewheat and 5% pea fiber samples were not different when overcooked (Table 3). The 10% pea fiber sample lost more material to cooking water. Iodine binding capacity of the wholewheat cooking water was lower than that of the control when samples were overcooked. Nonpolar lipid, originating from the wholewheat germ component, has been shown to reduce starch granule disruption during cooking of spaghetti (Matsuo et al., 1986) and may have helped prevent amylose leaching.

A second durum wheat (CWAD2) and a hard red spring (CWRS) wheat were tested to verify the main trends for CWAD1. Instron measurements indicated that the trends seen with the CWAD1 series were also evident for both the CWAD2 and CWRS samples (Tables 1&2) when cooked to optimum or when overcooked. The 2% xanthan gum supplemented noodles were firmer ($P < 0.05$) than controls in both cases. The 5% pea fiber and wholewheat samples were less firm than corresponding controls.

Dynamic rheometry measurements (Tables 1 and 2) corroborated the general trends seen in the first durum noodle series. The wholewheat samples of both CWAD2 and CWRS were firmer than other noodle samples in their respective series, as indicated by greater G'. Both wholewheat pasta η' values were larger in comparison to controls, xanthan gum and pea fiber containing samples. Results were consistent whether cooked to optimum or overcooked. Pasta containing 5% pea fiber were slightly less stiff than respective controls when cooked to optimum, as indicated by a lower G', while η' remained similar to the control. When overcooked both samples retained the same degree of rigidity and η' as their respective control sample. The 2% xanthan gum was not different from the control sample in either CWAD2 or CWRS when cooked to optimum. Differences occurred in G' and η' between the control and the 2% xanthan gum samples for both CWAD2 and CWRS when overcooked (Table 2).

Cooked weights, swelling index values and moisture content were not different within each group at both cooking times (data not shown), consistent with results of CWAD1. Cooking losses displayed the same general trends as for the CWAD1 series (Table 3). Pasta samples enriched with xanthan gum in both cases were not different in cooking loss from controls when cooked to optimum, but were lower in iodine binding capacity. Overcooking resulted in higher cooking losses without accompanying increases in amylose content of cooking water. CWRS wholewheat pasta lost more solids to cooking water while having lower iodine binding capacity than the control at optimum cooking. No difference was found between CWRS wholewheat and control pasta in total dissolved solids when overcooked. Again, wholewheat pasta leached less amylose into the cooking water. In contrast, samples containing pea fiber were not different from control samples at either cooking time.

CONCLUSIONS

ADDITION OF LOCUST BEAN GUM and especially xanthan gum improved pasta firmness characteristics. Pasta enriched with gums had increased tolerance to overcooking which was probably due to formation of a mechanical network surrounding the starch granules during cooking and subsequent gelatinization. In contrast, fortification of pasta products with pea fiber (particulate) seemed to disrupt the continuity of the protein-starch network, resulting in moderate reduction in pasta firmness. Pasta produced from wholewheat was moderately less firm than that of conventional pasta, as assessed by the Instron. Wholewheat pasta samples displayed development of a weak network which resulted in enhanced stiffness measured by small deformation mechanical tests. Small deformation and large deformation mechanical analyses should therefore be considered as complementary techniques, useful in elucidation of different types of network structures of starch based products.

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Ms received 7/12/94; revised 3/30/95; accepted 6/22/95.

Contribution No. 733 of the Canadian Grain Commission, Grain Research Laboratory, Winnipeg, Canada.

We gratefully acknowledge the expert technical assistance of D. Martin, J. Lachance, R. Daniel, and M. Lymych, and the financial support for parts of this study by the Natural Sciences and Engineering Research Council of Canada.

Inhibition of Horse Bean and Germinated Barley Lipoxygenases by Some Phenolic Compounds

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ABSTRACT

Phenolic compounds have been repeatedly implicated as potent antioxidants. Efficiency has been frequently estimated by radical scavenging activity and few reports have considered lipoxygenase (LOX) inhibition. Horse bean LOX was inhibited by a range of phenolic acids, gallates and flavonoids. All compounds tested were uncompetitive inhibitors with the exception of flavonol aglycons which were non competitive type. In each class of compounds, inhibition constants were strongly affected by structures. Inhibition patterns of (−)-epicatechin on germinated barley LOXs were detailed: (−)-epicatechin acted as an uncompetitive inhibitor while (−)-epicatechin reduced hydroperoxide formation by its radical scavenging activity and thus limited enzyme activation.

Key Words: antioxidants, barley, flavonoids, lipoxygenase, phenolic acids.

INTRODUCTION

LIPID OXIDATION has been reported as responsible for "cardboard off flavor" generated in stored beer (Angelino, 1991). According to Kobayashi et al. (1994), lipoxygenase (LOX) is the main factor initiating fatty acids oxidation during wort production. To reduce and delay this off flavor formation and therefore preserve food products, synthetic antioxidants such as sulfites or butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are extensively used. However, because of their possible potential as promoters of carcinogenesis (Imaida et al., 1983), as well as general consumer rejection of synthetic food additives, active investigation concerning the effectiveness of naturally occurring antioxidants are being carried out. Among these, phenolic compounds, especially flavonoids have been repeatedly implicated as active antioxidants (Pratt, 1990; Chimi et al., 1991; Maestro-Duran and Borja-Padilla, 1993). Maillard reaction products, widespread in processed foods, also have shown antioxidant activity (Elizalde et al., 1991).

Malt contains such natural antioxidants originating from barley (mainly phenolic compounds) or from the malting process (mainly Maillard reaction products). According to Jerumanis (1985) and Mc Murrogh and Byrnd (1992), barley polyphenols mostly include phenolic acids and their related esters (vanillic, *p*-coumaric, ferulic and hydroxycinnamic acids) and flavonoids (mainly flavonols, flavans and proanthocyanidins). An isoflavonoid, the 2"-*O*-Glycosylisovitexin from green barley leaves, has been reported as a potent antioxidative compound (Kitta et al., 1992; Osawa et al., 1992). However, if phenolic compounds have frequently been reported to act as quenchers of singlet oxygen (Mukai et al., 1991; Tournaire et al., 1993) and scavengers of various oxidizing radical species (Ricardo da Silva et al., 1991; Cotelle et al., 1992; Costantino et al., 1992; Tsujimoto et al., 1993; Hanasaki et al., 1994), few studies have considered their LOX inhibitory properties (Dohi et al., 1991; Lyckander and Malterud, 1992; Voss et al., 1992).

Thus, the main objective of this investigation was to assess phenolic compounds as LOX inhibitors in order to establish structure-antioxidative efficiency relationships and to elucidate the inhibition mechanism. Due to its high LOX content, horse bean was chosen as an enzyme source. Results were compared to those with the two LOXs isolated from germinated barley, the purification of which was previously reported (Hugues et al., 1994). Better knowledge of the inhibitory properties of barley endogenous polyphenols is essential to improving flavor stability of packaged beer.

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MATERIALS & METHODS

Materials

Tardenois Horse bean (*Vicia fabia* L.) flour was used as an enzyme source. Triumph barley (*Hordeum vulgare*) was malted in the IFBM (Institut Français des Boissons de la Brasserie Malterie) micromalting as described by Hugues et al. (1994). Fractogel butyl TSK 650 M was from Merck (Darmstad, Germany) and CM Sepharose CL6B from Pharmacia (Uppsala, Sweden). Chlorogenic acid and flavonoids were purchased from Extrasynthese (Genay, France), gallic acid and its esters from Fluka (Buchs, Switzerland). Phenolic acids, linoleic acid and all other chemicals were reagent grade supplied by Sigma (St Louis, MO).

Extraction and purification procedure

Horse bean and germinated barley LOXs were extracted according to Hugues et al. (1994). The two germinated barley LOXs (LOX1 and LOX2) were 18.3 and 44.5 fold purified in 3 steps, fractional precipitations by ammonium sulfate, hydrophobic chromatography with butyl TSK 650M (Merck) followed by ion exchange chromatography with CM Sepharose CL6B, as reported by Hugues et al. (1994).

Assay procedure

For routine LOX analysis, the substrate was 2.5 mM linoleic acid dispersed in phosphate buffer (0.1M, pH 6.5) containing Tween 20 (0.1%). LOX was assayed by polarography with a Clark electrode using an air-saturated substrate solution at 30°C. For inhibition studies with phenolic compounds, linoleic acid was varied from 0.1 to 5 mM in the control and with 3 concentrations of inhibitor. All assays were performed in duplicate. Apparent Km and Vm were determined by using a non linear regression data analysis program developed for the IBM PC by Leatherbarrow (1987). LOX activity was expressed as nanomoles of oxygen consumed/sec (nkat).

HPLC analysis

A Varian liquid chromatograph (9010 pump and 9050 detector driven by a 9020 Workstation) was used with detection at 210 (linoleic acid), 234 (hydroperoxide) or 280 nm ((−)-epicatechin).

Linoleic acid oxidation

For each reaction time tested, 0.5 mL of oxidized substrate (linoleic acid 2.5 mM supplemented with oleic acid 2.5 mM used as internal standard) was mixed with an equal volume of ethanol and immediately acidified to pH 2 with HCl 6N. Samples of 0.01 mL were injected into a Lichrospher RP18 endcapped (MERCK) reversed phase cartridge

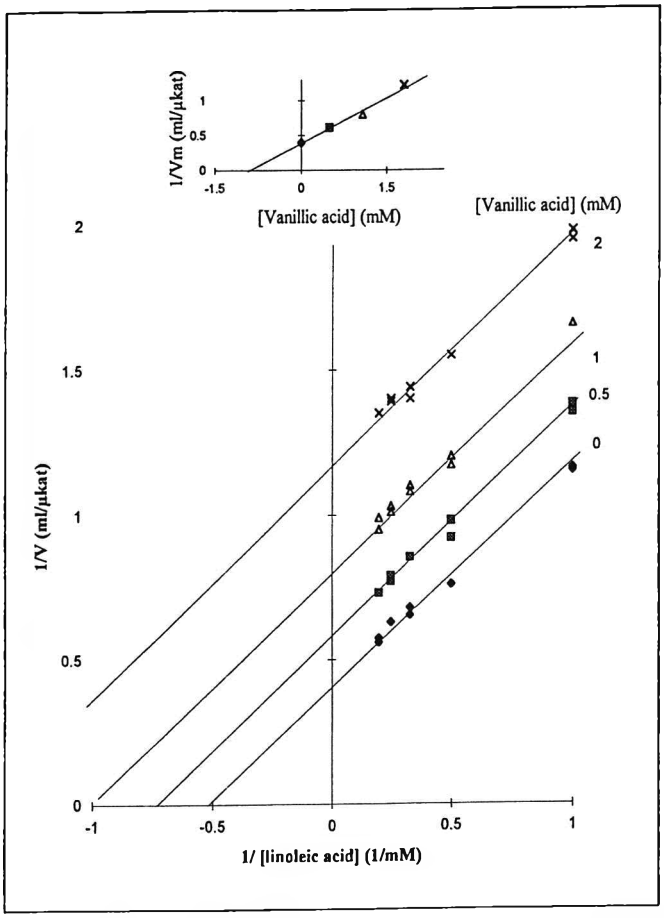


Fig. 1—Inhibition of horse bean LOX by vanillic acid. Insert: Effect of vanillic acid concentration on apparent 1/Vm.

Table 1—Inhibition constants and patterns of some phenolic acids with horse bean LOX as enzyme source

Type of substitution (relative to acid function)	Benzoic series	Ki (mM)	Cinnamic series	Ki (mM)
1 OH (ortho)	Salicylic acid	9.5		
1 OH (meta)	3-hydroxybenzoic acid	12.7		
1 OH (para)	4-hydroxybenzoic acid	7.5	Coumaric acid	8.0
1 OH (para)				
1 OCH3 (meta)	Vanillic acid	0.9	Ferulic acid	1.6
2 OH (ortho-meta)	Gentisic acid	4.5		
2 OH (ortho-para)	Resorcylic acid	6.5		
2 OH (para-meta)	Protocatechuic acid	0.6	Caffeic acid	1.1
3 OH (para-meta)	Gallic acid	0.9		

Table 2—Inhibition constants and patterns of some gallate esters with horse bean LOX as enzyme source

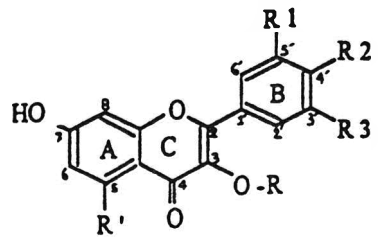
Inhibitor	Ki (mM)
Gallic acid	0.90
Ethylgallate	0.15
Propygallate	0.03
Octylgallate	0.08
Laurylgallate	0.10

(guard column 1 cm long and column 12.5 cm long, 4 mm i.d., particle size 5 μm). The gradient method used for separation was slightly modified from that described by Lee (1992). Solvents were (A) 0.1% trifluoroacetic acid (pH 2.6) and (B) 20% methanol mixed with 80% acetonitrile. The column was equilibrated at a flow rate of 1 mL min^{-1} with 80% B. The gradient profile was 0—7 min, 80% B; 7—18 min, 80—100% B and 18—22 min 100% B.

Reaction product specificity

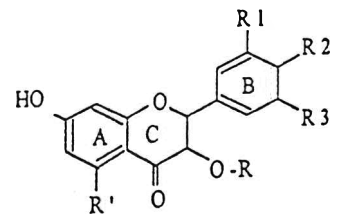
Extraction and HPLC separation of reduced hydroperoxides were performed according to Hugues et al. (1994).

Table 3—Structures, inhibition constants and patterns of flavonoids assessed as horse bean LOX inhibitors



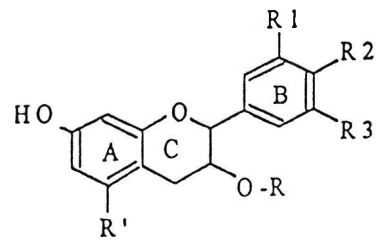
Flavonols

Inhibitor	R'	R_1	R_2	R_3	R	Ki (μM)	Inhibition patterr
Kaempferol	OH	H	OH	H	H	15	Non competitive
Quercetin	OH	OH	OH	H	H	9	Non competitive
Myricetin	OH	OH	OH	OH	H	23	Non competitive
Quercitrin	OH	OH	OH	H	Rhamnose	60	Uncompetitive
Isoquercitrin	OH	OH	OH	H	Glucose	65	Uncompetitive
Rutin	OH	OH	OH	H	Rutinose	80	Uncompetitive
Astragalin	OH	H	OH	H	Glucose	75	Uncompetitive
Fisetin	H	OH	OH	H	H	6	Uncompetitive



Flavanonol

Dihydroquercetin	OH	OH	OH	H	H	65	Uncompetitive



Flavan-3-ols

(−)-epicatechin	OH	OH	OH	H	H	1000	Uncompetitive
(+)-catechin	OH	OH	OH	H	H	500	Uncompetitive
Epigallocatechin	OH	OH	OH	OH	H	100	Uncompetitive

RESULTS & DISCUSSION

Inhibitory properties in relation to structure

Inhibition by phenolic acids. The inhibitory properties on horse bean LOX of the benzoic acid series and the corresponding cinnamic acid series were compared using linoleic acid as substrate. In each case, the type of inhibition was deduced from Lineweaver-Burk double reciprocal plots. Inhibition constants were calculated from secondary plots of apparent $1/V_m$ against inhibitor concentration. Parallel lines were obtained (Fig. 1) for vanillic acid. All aromatic carboxylic acids tested were uncompetitive inhibitors and therefore exhibited affinity for the substrate-complex enzymatic form; their apparent K_i were compared (Table 1). The lower the value of the apparent K_i, the greater the antioxidative efficiency. In the benzoic series, vanillic and protocatechuic acids were more potent inhibitors than their cinnamic acid counterparts. Cuvelier et al. (1992), Marinov and Yanishlieva (1992) and Shahidi and Wanasundara (1992) reported opposite results when antioxidative efficiency was estimated by free radical scavenging activity in apolar medium. They reported that the CH=CH–COOH (cinnamic acids) group ensured the highest antioxidative efficiency. In agreement with

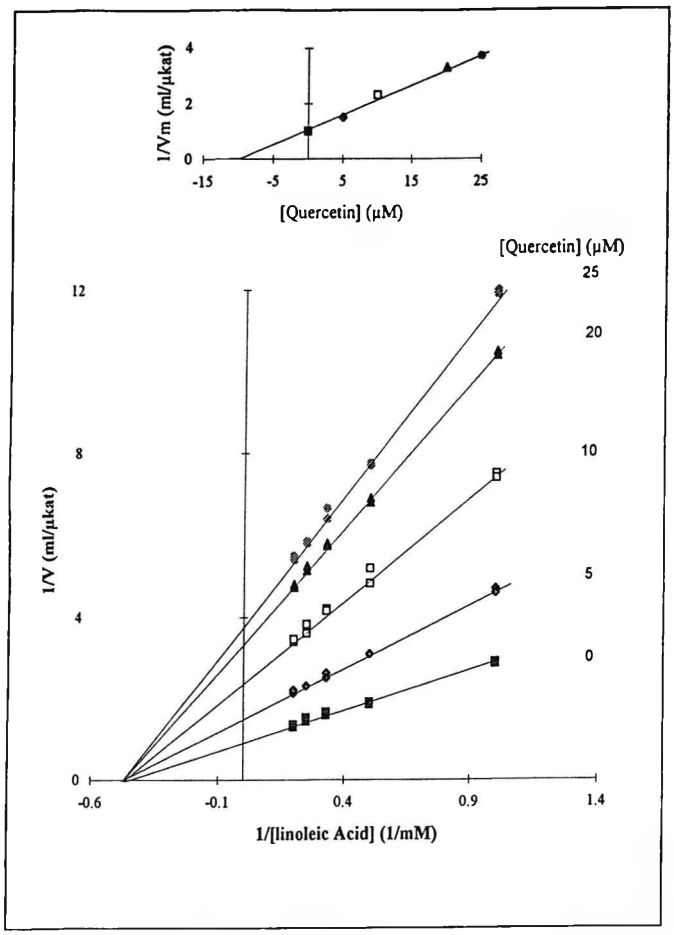


Fig. 2—Inhibition of horse bean LOX by quercetin. Insert: Effect of quercetin concentration on apparent 1/Vm.

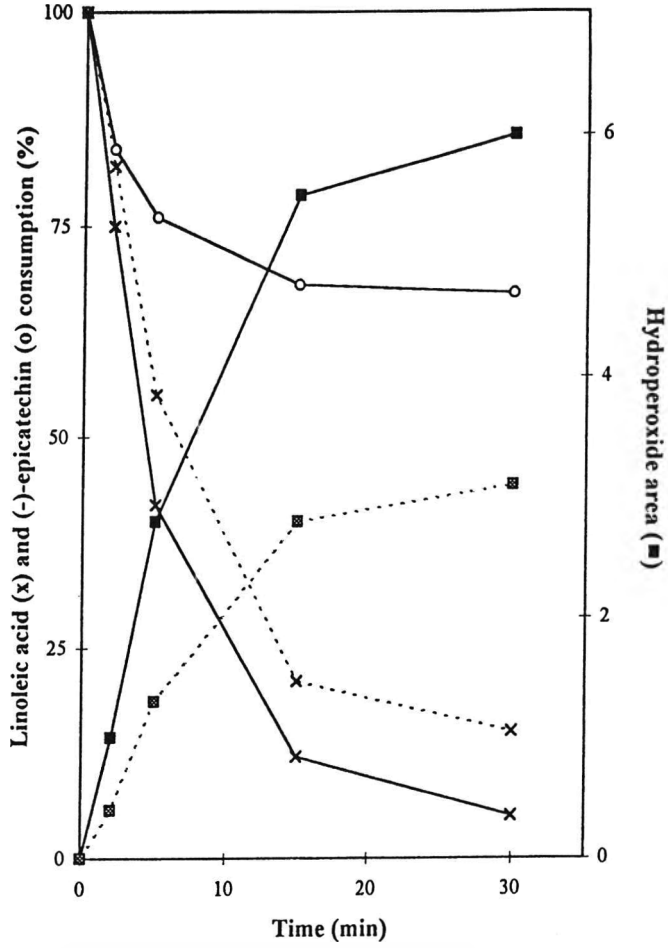


Fig. 3—Effect of (−)-epicatechin on the linoleic acid oxidation catalyzed by Barley LOX. Loss of linoleic acid, formation of hydroperoxide and loss of (−)-epicatechin. Full lines are for the blank experiment and dotted lines are for the experiment in the presence of (−)-epicatechin. Conditions: 2.5 mM linoleic acid was oxidized by 10 nkat.ml^{-1} of a partially purified germinated barley LOX extract, in the absence or the presence of (−)-epicatechin (2 mM).

Porter et al. (1989), their opposite findings could be the result of assay medium characterized by different polarity. In both series, the dihydroxy derivatives were more efficient than monohydroxy derivatives. Hence, protocatechuic and caffeic acids were more potent inhibitors than their monophenol counterparts, i.e. 4-hydroxybenzoic and *p*-coumaric acids. However, addition of a third hydroxyl group did not improve efficiency (protocatechuic acid > gallic acid). Comparison between the three monohydroxy benzoic derivatives showed that the *m*-substitution (3-hydroxybenzoic acid) led to the least antioxidative efficiency, in agreement with the findings that *m*-substituted derivatives had least chemical reactivity. Concerning diphenolic acids, the double substitution in the *m* and *p*-positions (protocatechuic acid) was linked to the lowest apparent K_i value. Our results also showed that the presence of one or two methoxy groups in the neighborhood of the hydroxyl greatly enhanced effectiveness (vanillic acid > 4-hydroxybenzoic acid, ferulic acid > *p*-courамic acid), in accordance with the results of Marinova and Yanishlieva (1992). Nevertheless, the methoxy substitution was almost equivalent to the addition of a second hydroxyl group (comparison between vanillic and protocatechuic acid). However, in terms of radical scavenging activity, protocatechuic acid was substantially more potent than vanillic acid (Cuvelier et al., 1992).

Inhibition by gallic acid and its esters. Gallate esters were also characterized as LOX uncompetitive inhibitors. Increasing chain length affected antioxidative efficiency (Table 2). Among the gallates esters tested, all were more potent inhibitors than gallic acid. However, increasing ester chain length resulted in decreased K_i only up to three carbons. Hence, propyl gallate appeared as the most potent antioxidant with a K_i value 30 times lower than that of gallic acid. With longer chains, antioxidative efficiency slightly decreased. These lower affinities for the substrate-complex enzymatic form could result from intrinsic hydrophobic interactions occurring in these long chains. These results did not support other findings concerning soybean lipoxygenase-2 inhibition, reported by Peterman and Siedow (1983). They suggested that the K_i value was directly linked to the lipophilicity of the inhibitor, the more lipophilic molecules being most inhibitory.

Inhibition by flavonoids. Some flavonols, flavanonols and flavan-3-ols (structures in Table 3) were tested as LOX activity inhibitors, in order to establish some structure-antioxidative efficiency relationships. Only flavonol aglycons showed a non competitive pattern and therefore exhibited affinity for both free and substrate-complex enzymatic forms. As an example, the non competitive pattern determined for quercetin by the Lineweaver-Burk plot is included (Fig. 2). All other compounds, in which rings A or C were modified, were uncompetitive inhibitors. These results suggested that the 3,5,7-trihydroxy-benzopyrane-4-one group is essential for affinity toward the free enzymatic form, in accordance with reports of Lyckander and Malterud (1992).

Concerning antioxidative efficiency, the flavonol aglycons and fisetin appeared as the most efficient inhibitors. Their apparent inhibition constants were more than 100 times lower than those associated with catechins. Comparison between quercetin and fisetin illustrated the weak effect exerted by the 5,7-hydroxylation of the A ring on antioxidative efficiency, as has been

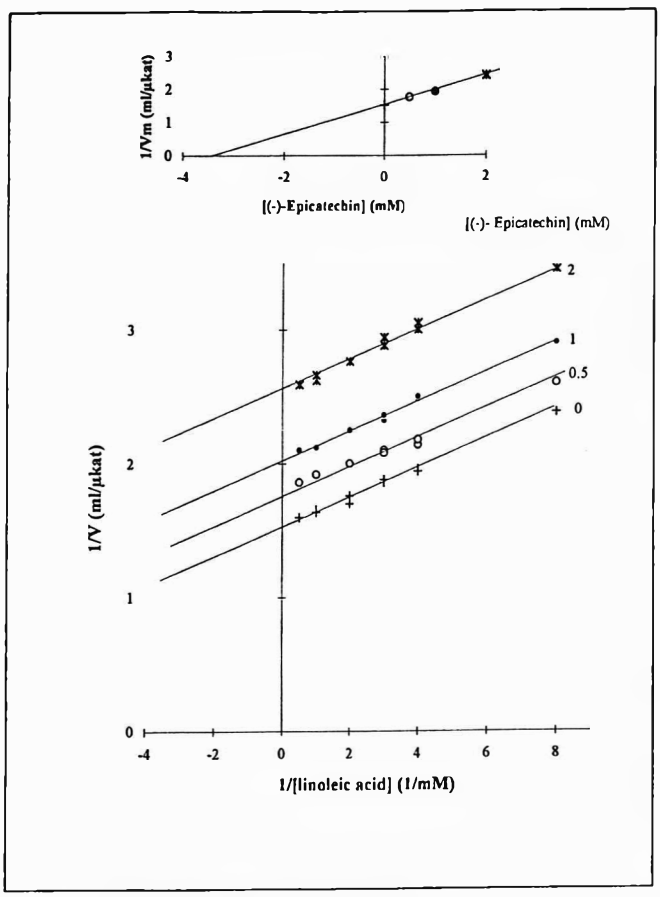


Fig. 4—Inhibition of germinated barley LOX1 by (−)-epicatechin. Insert: Effect of (−)-epicatechin on apparent 1/Vm.

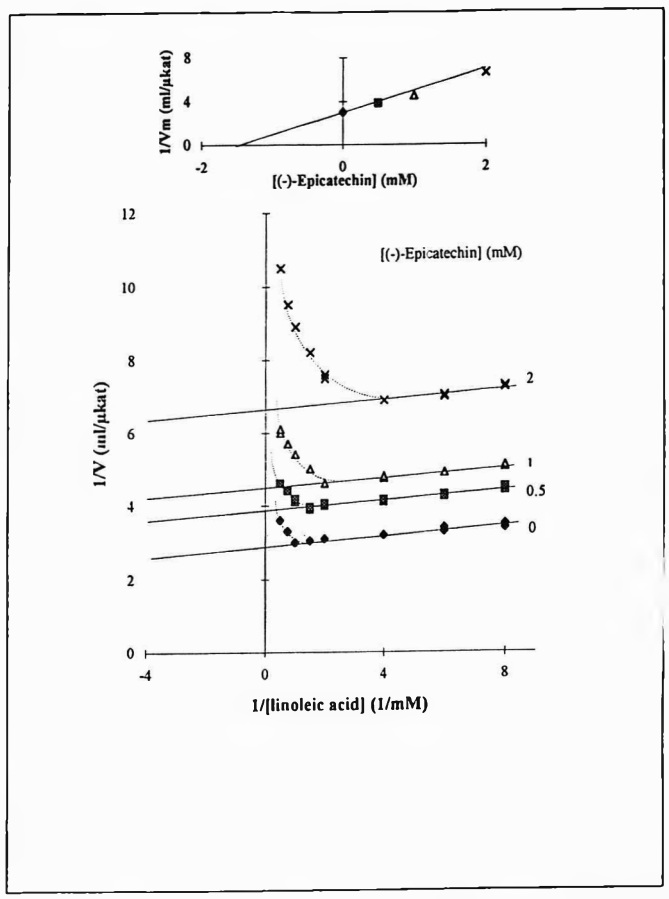


Fig. 5—Inhibition of germinated barley LOX2 by (−)-epicatechin. Insert: Effect of (−)-epicatechin on apparent 1/Vm.

reported by Shahidi and Wanasundara (1992). Conversely, glycosylation of the hydroxyl in C3 led to a large decrease of inhibition effect of LOX activity. The loss in antioxidative activity increased with complexity of the substituted sugar (quercitrin > rutin). Similarly, the lack of a double bond between C2 and C3 (flavonol > flavanonol) or the lack of a ketone group (flavanonol > flavan-3-ol) resulted in a strong increase of K_i value. These results were in agreement with findings of Krol et al. (1994). Comparison between mono-, di- and triphenols suggested that the presence of 2 hydroxyl groups enhanced antioxidative efficiency. However, if the addition of a third group led to a decrease in inhibition constant of flavan-3-ols (epigallocatechin > (−)-epicatechin), this was not confirmed for the flavonols (quercetin > myricetin). In addition, spatial configuration seemed also to affect antioxidative efficiency since (+)-catechin was two times more efficient than (−)-epicatechin. Thus, structure of ring C appears to be important in antioxidant activity. Similar results were reported by Tournaire et al. (1993) for efficiency of those compounds to quench singlet oxygen.

Inhibition of purified germinated barley lipoxygenases

Inhibition pattern of (−)-epicatechin. The inhibitory effect of (−)-epicatechin on the enzymatic oxidation of linoleic acid was monitored by HPLC at 210, 234 and 280 nm. The enzymatic extract was a partially purified extract of germinated barley lipoxygenase, i.e. eluted from hydrophobic chromatography (Hugues et al., 1994). Degradation of linoleic acid (2.5 mM) with and without (−)-epicatechin (2 mM) was compared (Fig. 3). In the presence of (−)-epicatechin, a weak inhibition of fatty acid oxidation was observed: initial degradation rate was about 70% of that noticed in the system without (−)-epicatechin. However, addition of (−)-epicatechin led to hydroperoxide levels two times lower than those observed when no phenolic compound was present. The difference in hydroperoxide formation rate could not be ascribed to lipoxygenase inhibition alone but resulted also from the radical scavenging activity of (−)-epicatechin (Hirose et al., 1991; Terao et al., 1994). A slight consumption of (−)-epicatechin occurred during the enzymatic reaction to attain a 70% residual level after 30 min oxidation. However no degradation products of (−)-epicatechin such as the "quasi-dimeric" ones described by Hirose et al. (1991) were observed in our chromatograms.

Inhibitory patterns of (−)-epicatechin on the two purified germinated barley LOXs (LOX1 and LOX2) were compared, with linoleic acid (0.125—2 mM) as substrate (Fig. 4 for LOX1; Fig. 5 for LOX2). Concerning LOX1, inhibition was of the uncompetitive type as determined by Lineweaver-Burk double reciprocal plots. The apparent inhibition constant calculated from the secondary plot (insert) was close to 3.5 mM. However, plots for LOX2, with the same substrate concentration range appeared parallel only for linoleic acid concentrations < 0.5 mM. For higher concentrations, they passed through a minimum and curved up as they approached the 1/v axis. The minima of the curves moved to higher rate and substrate concentration values as (−)-epicatechin concentration decreased. Similar shapes were described by Segel (1975) for enzyme activation with increasing activator concentrations, when substrate and activator bound to a single regulatory site. In previous studies (Hugues et al., 1994), we precisely observed an inhibition by excess linoleic acid and suggested that fatty acid and hydroperoxide were in competition for a single regulatory site on barley LOXs, in agreement with conclusions of Schilstra et al. (1992) and Wang et al. (1993). According to the previous hypothesis, LOX2 inhibition by (−)-epicatechin could be due to two mechanisms:

Addition of (−)-epicatechin in reaction media resulted in lower activator (hydroperoxide) levels leading to higher substrate/activator ratios and therefore a stronger inhibition by ex-

Table 4—Inhibition constants and patterns of some endogenous barley phenolic compounds and quercetin toward barley LOX1 and LOX2, and horse bean LOX

Inhibitor	Inhibition pattern	Ki/ LOX1 (mM)	Ki/ LOX2 (mM)	Ki/ horse bean LOX (mM)
chlorogenic acid	Uncompetitive	3.00	3.50	1.00
(−)-epicatechin	Uncompetitive	3.50	1.50	1.00
(+)-catechin	Uncompetitive	1.50	2.50	0.50
quercitrin	Uncompetitive	0.07	0.10	0.009
quercetin	Noncompetitive	0.06	0.04	0.06

cess linoleic acid, which also occurs for lower substrate concentrations. Concerning LOX1, the absence of inhibition by excess linoleic acid (in our conditions) could result from a higher affinity of the hydroperoxide to LOX1 regulatory site compared to that of linoleic acid.

(−)-epicatechin is an uncompetitive inhibitor. With linoleic acid concentrations <0.25 mM, for which no inhibition by excess substrate occurred (Fig. 5), parallel lines were observed, allowing us to calculate an apparent inhibition constant. This was estimated at 1.5 mM, i.e. more than twice lower than that associated with LOX1.

Inhibition properties of some barley endogenous polyphenols. Some barley endogenous phenolic compounds, i.e. chlorogenic acid (a hydroxycinnamic derivative), (−)-epicatechin and (+)-catechins (two flavan-3-ols) and quercitrin (a glycosylated flavonol) were tested as inhibitors of purified germinated barley LOXs (LOX1, LOX2). Inhibition patterns were also compared (Table 4) with that of quercetin (a flavonol aglycon). All compounds tested were efficient LOX1 and LOX2 inhibitors. Among these, only the flavonol aglycon was a non competitive inhibitor; the others were of the uncompetitive type. Similar results have been reported for horse bean LOX inhibition. Concerning apparent inhibition constants, apart for (−)-epicatechin, these were basically similar whatever the considered LOX. (−)-epicatechin showed more than two times higher affinity for LOX2, compared to LOX1 which has been reported to be mainly responsible for off flavor development in beer. Flavonols appeared as the most efficient inhibitors.

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Ms received 1/8/95; revised 4/12/95; accepted 5/12/95.

Thanks are due to the "Chambre syndicale de la Malterie Française," the "Brasseurs de France," and to the "Ministère Français de la Recherche" for financial support.

Dehydrated Precooked Pinto Bean Quality as Affected by Cultivar and Coating Biopolymers

H.L. SU and K.C. CHANG

ABSTRACT

The relationship of cultivars and coating treatment to quality of precooked dehydrated pinto beans was investigated. The pinto cultivars, studied were Fiesta, Othello, and Topaz, and 12 types of biopolymers, including modified starches, dextrins, maltodextrins, and alginate, were used as coating agents. Beans were blanched, soaked, steam cooked, coated by dipping in biopolymer solutions at 70°C, and dried in a convection oven. Eight biopolymers effectively reduced splitting of beans without detrimental effects on rehydration, firmness, or color. Cultivar had a strong effect on wholeness of bean products and Fiesta was best for dehydration. Residual trypsin inhibitor activity of precooked pinto beans was ~5%.

Key Words: pinto beans, dehydration, precooked, coating biopolymers

INTRODUCTION

BEANS are low-cost and nutritious but a major reason for underuse of beans is their prolonged cooking requirements. Development of quick-cooking precooked dehydrated bean products would be of advantage to consumers and food service institutions and could expand markets for beans.

Dehydrated precooked bean products have been investigated since the 1950s. However, most research had not included pinto beans. Feldberg et al. (1956) developed a "quick-cooking" pea, which could rehydrate in 5 to 10 min without additional cooking. In that process, the peas were frozen to minimize bursting before drying. The process was also satisfactory for Great Northern beans, but the need for a freezing step made the process uneconomical. Steaming for 15 min before soaking was used to destroy lipoxidase activity, ensure complete hydration, and improve storage stability of dehydrated peas and red kidney beans (Dorsey et al., 1961). Steinkraus et al. (1964) developed a process for producing "quick-cooking" dehydrated beans. The process involved soaking beans in water, precooking in steam, coating with gum, and dehydrating. The process was satisfactory for a many types of beans, including New York State pea, Perry marrow, and New York State red kidney. The processed beans were ready for consumption after covering with hot water and boiling 30 min. In this process, "butterflying" of beans during cooking and dehydrating was economically controlled (butterflying <11%) with coating agents, which contained 20% sugar, 0.5% alginate, or 5% gelatinized starch.

Thermal processing can destroy trypsin inhibitor activities. The residual trypsin inhibitor activity has been about 8% to 12% (Iyer et al., 1980), 10% to 15% (Estevez and Luh, 1985), 2% to 3% (Wang et al., 1988) or 13% (Dhurandhar and Chang, 1990) after processing.

Commercial pinto bean cultivars vary greatly in size and shape, which could affect dehydration. No studies have been reported on a suitable type of pinto bean for dehydration. Many biopolymers, such as modified starches, dextrins, and gums, have not been tested for effectiveness to improve product quality. Coating with biopolymers has improved the dehydration characteristics of carrots (Mudahar et al , 1989).

Our objective was to determine the effect of pinto bean cultivars and biopolymer treatment on quality of dehydrated precooked pinto beans.

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MATERIALS & METHODS

Materials

Three commonly grown pinto bean cultivars from Grand Forks, ND, were used. They differed in size and shape. Fiesta (large seed) was obtained from Fugleberg Seed & Bean Co., Othello (medium seed) from Grand Forks Bean Co., and Topaz (small seed) from Bergen & Kangaroo Co. Mixed pinto beans were obtained from a local elevator (Agrisales, Casselton, ND) for the coating study. Diameters of the seeds, length, width, and thickness, were measured using a vernier caliper (Bel-Art Products, Pequannock, NJ). Twelve kinds of biopolymers were used as coating agents before dehydration. The composition, source and concentrations used were as listed (Table 1).

Preparation of dehydrated precooked pinto bean

Pinto beans were washed and steam blanched at 100°C for 15 min (Steinkraus et al., 1964). They were soaked at 82.2°C for 1 hr and precooked with direct steam cooking at 100°C for 20 min. After precooking, beans were dipped in coating agents at 65 to 70°C for 5 min. Beans were dried in an air oven at 65°C for 5 hr.

Analyses

Moisture (AOAC 925.10, 1990). Dry bean samples were ground and passed through a 60-mesh screen. Ground beans (5 g) were dried in an air-circulated oven at 130°C for 2 hr. After cooling in a desiccator, the weight was determined and moisture content calculated.

Protein (AOAC 955.04, 1990). Crude protein content was measured. using the Kjeldahl method. The protein conversion factor of 6.25 was applied.

Splitting and butterflying. Beans were judged to be split if they had either a crack between cotyledons or a transverse fissure in the seed coat that was >2 mm. The beans with split seed coats or cracked cotyledons were counted. Results were reported as percent split beans. Butterflied beans were defined as those with two cotyledons separated by more than half of the length of the bean.

Color. The Gardner Lab Model XL-23 Tristimulus Colorimeter (Gardner Lab Inc., Bethesda, MD) was used to measure surface color of precooked dehydrated beans. The instrument was standardized with a standard white tile ($L = +91.94$, $a_L = -1.03$, $b_L = +1.14$).

Rehydration ratio. Rehydration ratio was determined after cooking 50g of dehydrated beans in 1000 mL tap water for 10 or 15 min. Rehydration ratio was calculated by dividing rehydrated weight by dry weight of the beans.

Firmness. After rehydration, the firmness of 100g precooked beans was measured, using an Instron Universal Testing Instrument (Model 1011, Instron Co., Canton, MA) equipped with a 500 kg weight beam and Kramer Compression-Shear cell. The speed was 20 mm/min. The peak height was recorded as the relative firmness of the bean (Wang et al., 1988).

Trypsin inhibitor activity. Trypsin inhibitor activity was determined, using the procedure of Kakade et al. (1974). Percent retention of the trypsin inhibitor activity was calculated based on the final activity in precooked pinto beans divided by the initial activity in the raw pinto beans.

Statistical analyses. Data were analyzed by one-way ANOVA, using the Statistical Analysis System (SAS Institute, Inc., 1985). Significant (p

Table 1—Coating agents

	Composition	Source	Concentration
N-lok	Modified starch	National Starch Co. (Bridgewater, NJ)	20%
Capsule	Modified starch	National Starch Co. (Bridgewater, NJ)	20%
Crystal gum	Dextrin	National Starch Co. (Bridgewater, NJ)	20%
K-4484	Dextrin	National Starch Co. (Bridgewater, NJ)	20%
Lo-Dex5	Dextrin	American Maize Co. (Hammond, IN)	20%
Lo-Dex10	Dextrin	American Maize Co. (Hammond, IN)	20%
Amaizo-5-starch	High amylose starch	American Maize Co. (Hammond, IN)	20%
Maltrin-040	Maltodextrin	Grain Processing Corp. (Muscatine, IA)	20%
Maltrin-100	Maltodextrin	Grain Processing Corp. (Muscatine, IA)	20%
Maltrin-200	Corn syrup solids	Grain Processing Corp. (Muscatine, IA)	20%
Keltone HV	Gelling grade sodium alginates	Kelco Division of Merck & Co. (Chicago, IL)	1%
Manugel DMB	Gelling grade sodium alginates	Kelco Division of Merck & Co. (Chicago, IL)	1%

< 0.05) difference comparisons were made, using Duncan's Multiple Range Test. Correlation coefficients among sizes, splits, rehydration ratios, and firmness were analyzed using Pearson's correlation.

RESULTS & DISCUSSION

Relation of cultivar to splitting and butterflying

The size diameters of three cultivars were compared (Table 2). The sizes (length × width × thickness, L × W × T) of the three cultivars differed ($p < 0.05$). The relation of different cultivars to splitting and butterflying of precooked pinto beans was compared (Table 3). Butterflying tended to decrease as bean size increased. Fiesta, the largest bean, had the lowest, and Topaz, the smallest, had the highest splitting and butterflying. The difference between Fiesta (the largest bean) and Othello (the medium bean) was not significant. Steinkraus et al. (1964) reported that 7% butterflied pea and 16% butterflied marrow bean occurred under similar processes. Estevez and Luh (1985) reported that red kidney bean had 59% splitting (dehydrated at air speed 9 m/min) and 84% splitting (dehydrated at air speed 165 m/min) and pinto bean 18.5% splitting (dehydrated at air speed 9 m/min) and 32.3% splitting (dehydrated at air speed 165 m/min).

Moisture and protein content

Moisture and protein contents of raw and processed pinto beans were compared (Table 4). Fiesta, the largest, had highest moisture content among raw beans. The moisture content of precooked pinto beans, like those of Estevez and Luh (1985), increased after processing. The protein contents of raw pinto beans were 21.3% to 22.5% and confirmed values reported by Fordham et al. (1975) and Wang et al. (1988). Estevez and Luh (1985) reported that 24% in red kidney and 22% in pinto beans occurred under similar processes. Processing did not affect protein content.

Effect of coating agents

Adding coating agents before drying affected splitting of precooked pinto beans (Table 5). N-lok (Modified starch), Capsule (Modified starch), Lo-Dex5 (Dextrin), Lo-Dex10 (Dextrin), Maltrin-40 (Maltodextrin), Maltrin-100 (Maltodextrin), Maltrin-200 (Corn syrup solids), and K-4484 (Dextrin) reduced the splitting of mixed pinto beans ($p < 0.05$). Dipping precooked beans in a 20% sucrose solution for 5 min was effective; sucrose, dextrose, and lactose appeared to give equal control of butterflying (Steinkraus et al., 1964). Steinkraus et al. (1964) also found butterflying could be reduced by adding alginates to the soaking water. Jen et al. (1989) hypothesized that biopolymers, which can penetrate into intercellular spaced and cell walls, may decrease the collapse of vegetable cells during dehydration.

Table 2—Diameter of seed size of pinto bean cultivars

Cultivar	Length (mm)	Width (mm)	Thickness (mm)	L × W × T (cm^3)
Fiesta	13.35[a] (0.53)	9.07[a] (0.23)	5.29[a] (0.39)	0.64[a] (0.06)
Othello	12.74[b] (0.85)	8.49[b] (0.61)	5.33[a] (0.37)	0.58[b] (0.09)
Topaz	12.61[b] (0.70)	7.93[c] (0.49)	5.01[b] (0.38)	0.51[c] (0.08)

[a-c] Means of 50 replicates. Means within same column not followed by same letter significantly different ($p < 0.05$).

Table 3—Relation of cultivar to splitting, rehydration, and firmness of precooked pinto beans

Cultivar	Butterflying (%)	Total splitting (%)	Rehydration ratio[c] 10 min	Rehydration ratio[c] 15 min	Firmness (kg force/100g bean) 10 min	Firmness (kg force/100g bean) 15 min
Fiesta	3.6[b]	13.4[b]	2.1[a]	2.1[b]	192.5[a]	161.0[a]
Othello	4.6[b]	16.6[b]	2.1[a]	2.1[ab]	196.3[a]	150.0[a]
Topaz	9.0[a]	34.3[a]	2.2[a]	2.2[a]	247.5[a]	190.0[a]

[a-b] Means of two replicates. Means within the column not followed by same letter significantly different ($p < 0.05$).
[c] Rehydration ratio expressed as g rehydrated weight/g dry precooked bean product.

Table 4—Moisture and protein contents of raw and precooked pinto beans

Cultivar	Moisture (%) Raw	Moisture (%) Precooked	Protein (%) Raw	Protein (%) Precooked
Fiesta	10.9[a]	14.0[a]	21.5[b]	20.4[b]
Othello	7.9[b]	13.8[a]	21.5[b]	20.7[ab]
Topaz	7.6[b]	12.5[b]	22.5[a]	21.3[a]
Mixed	7.3[b]	12.2[c]	21.3[b]	20.7[ab]

[a-c] Means of two replicates. Means within same column followed by different letter significantly different ($p < 0.05$).

Table 5—Effects of coating agents on splitting of precooked pinto beans

	Butterflying (%)	Total splitting (%)
Without coating	41.02[a]	61.39[b]
Coating with		
Crystal gum	32.65[b]	61.26[b]
N-lok	23.63[cd]	51.06[cdef]
Capsule	23.69[cd]	55.61[c]
Amaizo-5-starch	27.32[c]	60.82[b]
Lo-Dex5	15.82[f]	52.55[cde]
Lo-Dex10	16.39[ef]	52.79[cde]
Maltrin-040	21.56[de]	54.38[cd]
Keltone HV	35.05[b]	69.46[a]
Maltrin-100	17.26[ef]	46.56[f]
Maltrin-200	21.63[de]	48.20[ef]
K-4484	19.53[def]	49.05[def]
Manugel DMB	40.14[a]	69.14[a]

[a-f] Means of two replicates. Means within same column not followed by same letter significantly different ($p < 0.05$).

Rehydration ratio

No differences occurred in rehydration ratio among different cultivars after 10 min boiling (Table 3). Topaz had the highest rehydration ratio after boiling for 15 min. The rehydration ratios ranged from ~2 to 2.3. Bean size (L × W × T) correlated

Table 6—Trypsin inhibitor activities (TIA) of raw and precooked pinto beans

Cultivar	Raw (TIU/g)	Precooked (TIU/g)	Retention of TIA[b] (%)
Fiesta	25250[a] (a)	1346[a] (b)	5.3
Othello	20450[a] (a)	1226[a] (b)	6.0
Topaz	29988[a] (a)	1298[a] (b)	4.3
Mixed	30025[a] (a)	1427[a] (b)	4.8

[a] Means of two replicates. Means within same column followed by different letter significantly different ($p < 0.05$). Means within same row followed by different letter in parentheses significantly different ($p < 0.05$).

[b] Percent retention of trypsin inhibitor activity (TIA) calculated based on amount in raw pinto beans using the equation:

$$\% \text{ Retention} = \frac{\text{Trypsin Inhibitor (Precooked Pinto Beans)}}{\text{Trypsin Inhibitor (Raw Pinto Beans)}} \times 100\%.$$

negatively with both rehydration ratios after 10 and 15 min of boiling ($r = -0.99$, $p < 0.05$, for both rehydration ratios of the three cultivars). The dehydrated beans without coating and coated with crystal gum, Keltone HV, Manugel DMB, seemed to give higher rehydration ratios (2.20, 2.18, 2.30, 2.31). Correlations between rehydration ratios with butterflying ($r = 0.73$ and 0.69 for 10 and 15 min boiling, $p < 0.05$) were positive. Thus, split beans might have allowed cotyledons to take up more water during rehydration.

Steinkraus et al. (1964) found that the rehydration ratio was 2.0 for dehydrated pea beans after rehydrating 10 min in boiling water, soaking in water at 27°C for 5 hr, dipping in 20% sucrose syrup, and drying at 93°C for 50 min. It was 1.7 for pea bean, after soaking in water at 82°C for 40 min, dipping in 3% microfine sucrose, and drying at 93°C for 80 min. Rehydration ratios for the dehydrated pea beans after boiling for 15 min were ~2.1 and 1.9 (Steinkraus et al., 1964).

Firmness of precooked pinto beans

Firmness among different cultivars ($p < 0.05$) (Table 3) after boiling for 10 min or 15 min did not differ. Boiling for 15 min produced lower firmness than 10 min boiling. Both Fiesta and Othello had firmness values in the palatable range for cooked pinto beans. Firmness was high for beans treated with and without coating agents after rehydrating in boiling water for 10 min (firmness range: 150–200 kg/100g bean) than in boiling water for 15 min (firmness range: 97–177 kg/100g bean). However, only Maltrin-200 treated beans had a firmness higher than the palatable value after boiling 15 min. Canned beans with firmness <162 kg/100g for pinto beans were acceptable (Wang et al., 1988). Therefore, cooked beans showing firmness of 162 kg/100g pinto beans or lower were considered palatable or acceptable. Firmness of rehydrated beans after boiling for 10 min correlated negatively with rehydration ratios ($r = 0.72$, $p < 0.05$). However, firmness of beans rehydrated for 15 min did not correlate with rehydration ratios.

Color

The difference between coating ($L = 22 \sim 24$, $a_L = 9 \sim 11$, $b_L = 7.6 \sim 8.7$) and noncoating agents ($L = 24.33$, $a_L = 10.22$, $b_L = 8.41$) was negligible, except for Amaizo-5-starch ($L = 35.16$, $a_L = 6.53$, $b_L = 6.24$). The precooked pinto bean treated with Amaizo-5-starch became whiter (increased L-value), less red (decreased in a_L-value), and less yellow (decreased in b_L-value) than beans treated with or without coating agents.

Trypsin inhibitor activity

The trypsin inhibitor activities of precooked pinto beans were reduced by heat processing to ~4.3% to 6% of original values (Table 6). The residual trypsin inhibitor activity was 10% to 15% in dehydrated red kidney and pinto beans (Estevez and Luh, 1985), 2% to 3% in canned navy and pinto beans (Wang et al., 1988), and 3% in navy and kidney beans cooked at 100°C for 30 min (Dhurandhar and Chang, 1990).

CONCLUSION

CULTIVAR was strongly related to splitting of precooked pinto beans. Fiesta and Othello were good cultivars to produce dehydrated precooked pinto beans. Processing did not change the protein content of precooked pinto beans. Heat treatment inactivated >95% of trypsin inhibitor activity. Coating with biopolymer agents before drying reduced splitting and butterflying without detrimental effects on rehydration, firmness, or color. The blanching (100°C, 15 min), soaking (82.2°C, 1 hr), cooking (100°C, 20 min), coating (70°C, 5 min) and dehydration (65°C, 5 ~ 6 hr) conditions may provide a base for future pilot-scale studies of dehydrated precooked pinto bean products.

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Ms received 12/12/94; revised 6/9/95; accepted 6/22/95.

Presented at the 54th Annual Meeting of the Institute of Food Technologists, Atlanta, GA, June 25–29, 1994.

North Dakota Agricultural Experiment Station Journal Article No. 2321.

North Dakota Agricultural Products Utilization Commission, NDSU Agricultural Experiment Station, and the USDA-CSRS Alternate Crop Utilization Research Program supported this project ND 2705.

Texture of Pecans Measured by Sensory and Instrumental Means

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ABSTRACT

The texture of pecans (*Carya illinoensis*) from four cultivars (Barton, Mahan, Western and Wichita) was analyzed using sensory and instrumental methods. Sensory hardness, flexibility and crispness were rated by trained panelists. Ranking of hardness and crispness was also carried out. A Texture Analyser TA.XT2® was used for 50% compression, texture profile analysis (TPA), puncture and bending. Puncture and 50% compression gave best reproducibility, least variability and agreement with sensory data. The most relevant TPA parameters were cohesiveness, elasticity and fracturability. Parameters from the bending test did not indicate texture of the pecans suitably.

Key Words: pecans, sensory, compression, texture profile analysis

INTRODUCTION

A NUT IS A FRUIT SEED enclosed in a leathery or woody covering (the pericarp) from which it is usually separable (Grolier, 1992). For consumers, nuts are edible oilseeds of several different species, that have pleasant sensory properties. In many countries pecans (*Carya illinoensis*) are the most common nuts. The acceptance of pecans is due mainly to flavor and texture, and because they can be used in many dishes as a substitute for other nuts without diminishing flavor (Rombauer and Rombauer-Becker, 1973). Also, pecans can be cracked easily, whereas some other nuts have extremely hard shells (Grolier, 1992).

The texture of pecans is critical for acceptance (Bourne, 1982). This attribute is important for food processors, since the quality of their products may depend largely on texture of raw materials (Brennan et al., 1976). Pecans are not so oily as macadamia nuts; added to baked goods or confectionery products they also provide crunch (Rombauer and Rombauer-Becker, 1973; Mallos, 1986).

Although texture of pecans is important, there are not many reports about it. Apart from general statements on the importance of texture in nuts (Kramer and Twigg, 1972), or about crispness (Bourne, 1976; 1982.a), little published data has been related to the texture of nuts. Clark et al. (1977) studied some viscoelastic and macro nutrient characteristics of peanuts. They evaluated the uniaxial compression modulus in an Instron Universal Testing Machine. The oil content correlated significantly with the modulus evaluated at 3 sec and the ratio of moduli, but not with other moduli. Protein content correlated with the modulus evaluated at 5 sec. Another 12 correlations corresponded to content of various aminoacids. Results did not indicate a practical method that could be used for quality control or other texture measurements. Romo de Vivar and Brennan (1980) investigated the texture of peanuts, comparing measurements by instrumental methods. They also evaluated effects of frying ("oil roasting") on texture. Individual compression, a snap test and shear exhibited best reproducibility and showed changes resulting from processing. Textures of peanuts and pecans are not very similar, since peanuts are more homogeneous. Peanuts are legumes, whereas pecans are true nuts (Grolier, 1992) with different characteristics, such as cohesiveness and brittleness. Pecans are larger and people bite through the nut, using the incisor teeth, whereas peanuts are small and several seeds may be eaten at a time, grinding them with the molar teeth. This first impression is very important in detection and description of texture since there is an order of apparition for detection of textural properties (Bourne, 1982): touch, first bite, second bite, chewing, swallowing and residual sensations (Anzaldúa-Morales, 1994). An instrumental method (especially empirical techniques) must reflect considerations of what people do when they eat the food (Bourne, 1982, 1993).

Sensory and objective measurements were carried out (Resurreccion and Heaton, 1987) on early harvested and traditionally harvested pecans. A trained panel rated samples by category scaling for texture, color, appearance, flavor, and consumers carried out tests using 5-point hedonic scales. Instrumental measurements were made using an Instron Universal Testing Machine for puncture and shear. Some aspects of the instrumental analysis were not very clear. A 3.2 mm diameter punch was used for puncture test. This size is not very suitable for pecans since the arrangement is not close to a semi-infinite geometry (Bourne, 1982), which is a requirement for a good puncture test. Many samples would break before being cleanly penetrated by the punch. When we attempted to carry out similar measurements on Mexican cultivars, a smaller diameter punch had to be used, otherwise most kernels would break before completion. Not many indications were reported about conditions for the shear test, whether the samples were on a flat surface or rested on supports or if the blade passed through a slot. Preliminary tests we carried out showed that a sharp blade would be better than a blunt one. Measurements obtained by shear with a single blade did not have very good reproducibility.

Hung and Chinnan (1989) used a Warner-Bratzler blade, compression and a modified Kramer shear cell for measurement of texture of whole and chopped peanuts. Sensory assessments of crispness and crunch were compared. They concluded that the modified shear cell was the most sensitive to quantify textural quality of whole or chopped peanuts.

Pecans are a main product of Chihuahua state in Mexico. The most important cultivars, based on numbers of trees, are: Western Schley, Mahan. Wichita, Bradley, Barton, San Saba, Burkett, Stuart, Success and Desirable (CONAFRUT, 1974). Several nut producers plan to establish processing plants for pecans, as well as their products. They require a suitable instrumental method for characterization of pecan texture. Such information may be useful for quality control, improvement of cultivars, new product development and process design. Our objective was to determine the texture of pecans from four cultivars (Western Schley, Wichita, Mahan and Barton) by means of 50% compression, Texture Profile Analysis (TPA), puncture and bending. Sensory hardness, flexibility and crispness were rated by means of interval scales and hardness and crispness were determined by ranking tests, to verify instrumental results.

MATERIALS & METHODS

Sensory evaluation

Scoring for hardness, crispness and flexibility was carried out by means of 5-point interval scales, with end point anchors from "least" to "most" for each attribute. Ten judges (six women and four men),

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Table 1—General characteristics of pecan nut cultivars studied (BANSA, 1969).

Cultivar	Size	Flavor	Shell	Yield	Harvest
Barton	Medium	Good	Thin	Good	Late spring
Mahan	Very large	Weak	Very thin	Poor	Early
Western Schley	Long	Very good	Very thin	Good	Early
Wichita	Medium/ Large	Good	Thin	Very good	Late

Table 2—Proximate analyses (per 100g) of pecan nut cultivars (Espino-Rojas and Rodríguez-Tristán, 1982)

Cultivar	Moisture (%)	Ash (g)	Crude fibre (g)	Lipids (g)	Carbohydrates (g)	Protein (g)
Barton	4.61	0.78	24.99	8.57	25.95	35.1
Mahan	1.81	5.02	13.57	6.52	30.48	42.6
Western Schley	5.74	3.77	21.57	5.63	25.99	37.3
Wichita	5.06	5.59	15.07	11.02	28.46	34.8

Table 3—Mean sensory[c] ratings of pecan nuts

Attribute	Cultivar			
	Barton	Mahan	Western Schley	Wichita
Hardness	2.2a	2.3a	2.3a	2.2a
Flexibility	1.3a	1.2a	1.3a	1.1a
Crispness	3.8a	3.3a	3.9a	1.6b

[a,b] Means of a sensory attribute within a row with no common superscripts differ significantly ($p < 0.05$; $n = 8$).

[c] 5-Point scales were anchored from lowest to highest intensity.

composed of graduate students and department staff, were trained for rating with scales. Ranking tests were used (Larmond, 1977; Anzaldúa-Morales, 1994) for assessment of hardness and crispness. Fifteen trained panelists (ten male and five female judges) of department staff and graduate students, carried out ranking tests. Their training consisted of explanation of the purpose of the tests, method to apply, and properties to consider for ranking of samples. They practiced using selected samples of varying degrees of texture. They had been previously selected and trained for textural tests such as TPA, and knew the procedure for texture appraisal: touch, first bite, second bite, chewing, swallowing and residual stages; and were well acquainted with the meaning of textural terms. Each judge received four samples/session, and each sample consisted of two nut halves in a coded plastic custard cup. Each sample was evaluated three times.

Instrumental texture evaluation

All instrumental tests were carried out using a Texture Analyser TA.XT2® (Texture Technologies Corporation, Scarsdale, New York/Stable Micro Systems, Haslemere, Surrey, UK). To aid in selection of instrumental methods, eight trained panelists were asked to describe the texture of pecans and, as recommended by Bourne (1993), observations were made about actions they performed in order to judge the texture. For instance, they considered biting, chewing, bending, compressing between the thumb and the index finger, etc. Preliminary trials were carried out to determine which methods might be suitable. Four techniques were selected: Texture Profile Analysis (TPA), 50% compression, puncture and bending.

Texture profile analysis. Cylindrical samples were prepared from the nuts using a cork borer. Nut halves were placed on a flat surface and a cork borer was driven manually perpendicular to the sample. The cylinders were 3 mm in diameter and were cut to a standard length of 5 mm. A flat disk (50 mm diameter) was driven at 0.5 mm sec^{-1} onto individual cylinders (placed horizontally on the platform of the machine) in order to compress them to 50% of their diameter (final thickness 1.5 mm). A second compression was carried out with a 1 sec waiting time between both compressions. Fifteen replicates were tested.

50% compression. The shape of pecans is irregular, therefore, the compression of nut halves gave readings with high coefficients of variation. The test was carried out on cylindrical samples, using the same conditions as for TPA, but compressing each sample only once. The peak force was recorded for 15 replicates.

Puncture. A cylindrical flat-end punch (1 mm diameter) was used. The speed was 5 mm sec^{-1} and the punch was driven completely through the nut halves (8 mm travel). The peak force was recorded for 20 replicates.

Bending. Nut halves were placed on two vertical supporting bars with rounded upper ends (2 mm radius) separated 20 mm. A third bar with the same dimensions was driven perpendicular to the sample at 0.8 mm sec^{-1} with 4 mm travel. Fifteen replicates were used, and the first peak force, the final force, the area of the first peak and the distance for the first peak were recorded.

Statistical analysis

The data from sensory ratings and instrumental tests were subjected to analysis of variance using Systat 5.0 in Macintosh computers (models LCII and Quadra 630). The least significant differences were determined by means of Tukey's test (Anzaldúa-Morales, 1994). The data from ranking tests were transformed using Fisher and Yates' tables (Larmond, 1977; Anzaldúa-Morales, 1994) and subjected to analysis of variance and Tukey's test.

Materials

Four pecan cultivars were used: Barton, Mahan, Western Schley and Wichita. Their general characteristics (Table 1, BANSA, 1969), and proximate analysis (Table 2, Espino-Rojas and Rodríguez-Tristán, 1982) were compared.

RESULTS & DISCUSSION

Sensory evaluation

Sensory ratings for hardness, flexibility and crispness were compared (Table 3). For hardness and flexibility, no differences were found among cultivars. For crispness, only Wichita was less crisp than the others ($p < 0.05$). Nevertheless, some panelists could differentiate cultivars, as some preliminary tests showed. The judges had been asked to differentiate samples by paired comparisons and triangular tests. In most cases, they were able to differentiate Mahan and other cultivars ($p < 0.001$) and, in some cases, Barton. They could not distinguish between Western Schley and Wichita.

The ranks were transformed to numerical values by means of Fisher and Yates' tables (Larmond, 1977). Values assigned for the first (lowest intensity), second, third and fourth (highest intensity) ranks were -1.03, -0.30, $+0.30$ and $+1.03$, respectively. Averages of numerical values from transformation by the tables do not indicate a magnitude of sensory properties but show degrees of difference between cultivars (Anzaldúa-Morales, 1994). Hardness rankings indicated that the hardest cultivar was Mahan, notably harder than the others ($p < 0.0001$). Mahan had higher crispness ($p < 0.05$) than the other cultivars and Wichita was least crisp. No difference occurred between Barton and Western Schley.

Instrumental measurements

Some typical curves for puncture were compared (Fig. 1). Results from puncture, compression and bending were compared (Table 4). The effect of cultivar on puncture force was very marked ($p < 0.0001$). The coefficient of variation of the readings reached 18%. Mahan was harder than all others, but there was no difference among the others. This agreed with sensory evaluation.

As for puncture, the effect of the cultivar on compression (50%) was very strong. However, coefficients of variation were somewhat higher than for puncture (average coefficient of variation, 19%). The peak force for compression differentiated Mahan and Western Schley from Wichita and Barton, but did not

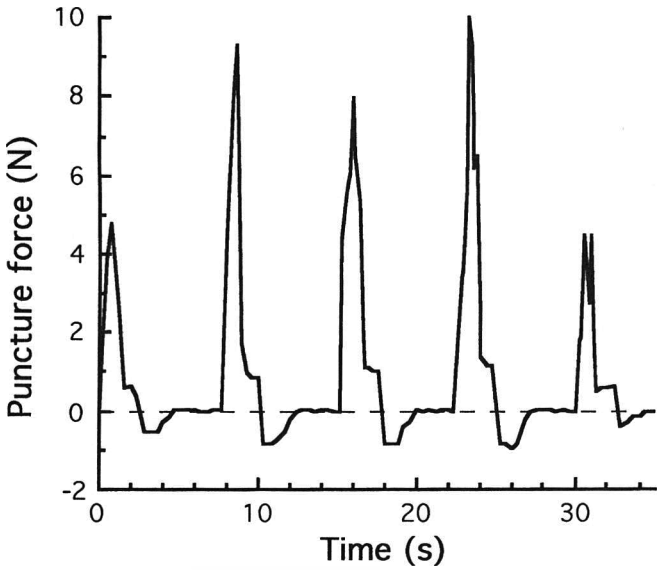

Fig. 1—Typical force (in Newtons) vs time curves for puncture of Mahan pecans.

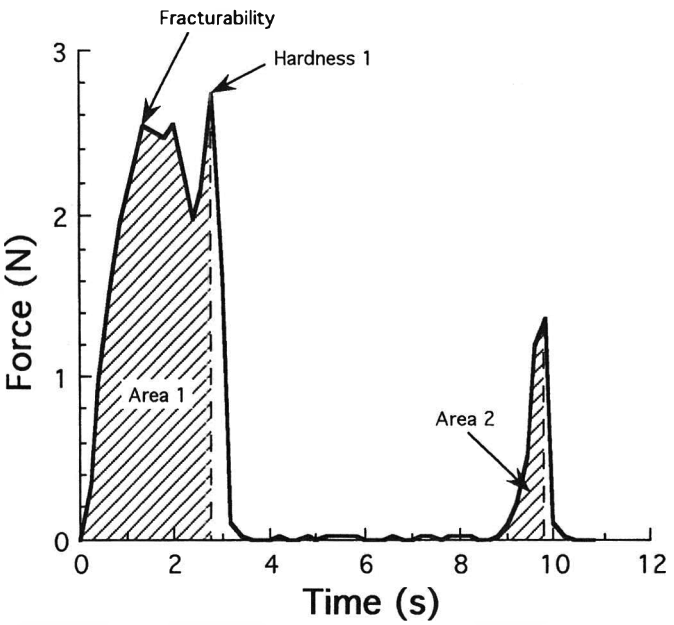

Fig. 2—Typical force (in Newtons) vs time curve for Texture Profile Analysis of Wichita pecans, showing some textural parameters. The test was performed on cylinders (3 mm diam, 5 mm long) placed horizontally in the machine and compressed to 50% of the original diameter.

Table 4—Averages of instrumental parameters from puncture, 50% compression and bending tests carried out with a Texture Analyser TA.XT2®

		Cultivar			
Test	Parameter	Mahan	Barton	Wichita	Western Schley
Puncture	Peak force, N	8.12a	6.47b	6.20b	6.08b
50% Compression	Peak force, N	1.37a	0.99b	1.08b	1.32a
Bending	First peak force, N	4.17a	5.39a	6.32a	0.91a
	First peak distance, mm	0.30a	0.84a	0.58a	0.38a
	Second peak force, N	26.4a	24.1a	23.4a	14.1b
	Second peak distance, mm	2.33a	2.86a	1.61b	1.70ab
	Area, N sec	34.0a	27.9ac	25.7c	13.5b
	Slope, N sec^{-1}	9.49a	9.81a	9.29a	6.80b

[a,b] Means of a texture parameter within a row with no common superscripts differ significantly ($p < 0.05$).

Table 5—Parameters of Instrumental Texture Profile Analysis (carried out with a Texture Analyser TA.XT2® of pecan nuts

	Cultivar			
Parameter	Barton	Mahan	Western	Wichita
Springiness, mm	0.39a*	0.42a	0.40a	0.42a
Cohesiveness	0.241ab	0.272a	0.243ab	0.216b
Chewiness	0.093bc	0.154a	0.130b	0.100c
Gumminess	0.235b	0.369a	0.323a	0.222b
Fracturability, N	0.94a [60%]**	1.23b [46%]	1.06ab [73%]	0.95b [93%]
Area 1, N sec	1.63b	2.33a	2.33a	2.04a
Area 2, N sec	0.38b	0.61a	0.57a	0.44b
Hardness 1, N	0.99b	1.37a	1.32a	1.08b
Hardness 2, N	0.77b	1.11a	1.06a	0.84b

* Mean in a row with the same letter are not significantly different.
** Values in brackets represent the frequency of curves exhibiting fracturability.

show any differences between Mahan and Western Schley or between Wichita and Barton. Puncture indicated that only Mahan was harder than the others, but compression showed that Mahan and Western Schley were firmer than the others.

In general, both instrumental methods agreed with sensory evaluations. Puncture had the advantage of being carried out more easily since the test was performed on nut halves. For compression cylindrical samples had to be taken from the pecan halves.

A typical Texture Profile Analysis curve is shown (Fig. 2). Averages for several parameters were compared (Table 5). TPA provides information about several parameters, and the data showed that Mahan had the highest magnitude for all parameters, whereas Barton seemed to be lowest in most attributes. Adhesiveness did not appear, except in two or three samples. This was expected since pecans are not sticky. No differences were observed for springiness; which, on average, was ≈0.40 mm for the four cultivars.

Wichita had the lowest cohesiveness (ratio of Area 2/Area 1) and differed from Mahan. No difference occurred in this property with Barton and Western Schley. The patterns for chewiness and gumminess were very similar: Mahan had the highest magnitude of both parameters and Wichita the lowest.

Not all curves exhibited fracturability. From 15 TPA curves for Barton, nine showed fracturability; and for the other cultivars, except Wichita, the frequency was relatively low. The frequency of curves with fracturability, as well as the average of the fracturability force, coincided with the observed ranking for crispness. However, due to the small number of paired data, regression analysis could not be applied. In another experiment regarding textural changes of pecans during roasting at different temperatures (Ocón and Anzaldúa-Morales, 1995), a good agreement was observed between fracturability force and crispness values and rankings.

Hardness 1 and hardness 2 had similar patterns; distinguishing Mahan and Western Schley from Barton and Wichita. Area 1, the work for the first compression, differentiated Barton from the three others. Area 2, the work for the second compression, had the same pattern as the two types of hardness. Generally, the value of Area 1 is considered more important than Area 2 since it corresponds to compression of an intact body. The second peak of the curve represents material that has undergone irreversible physical changes. Area 1 indicated that Barton was less resistant than the others. This seemed to show a different pattern than sensory appraisals. However, sensory rankings indicated that Mahan was harder than the other cultivars but did not differentiate Barton as the softest cultivar. The TPA primary parameters (hardness and compression work) indicated that Ma-

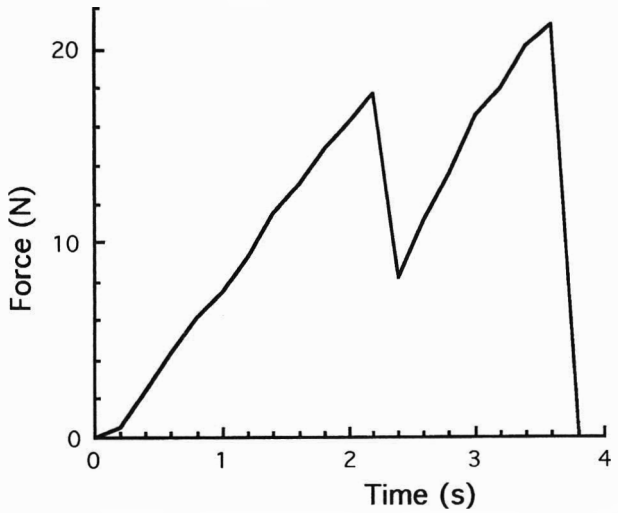

Fig. 3—Typical force (in Newtons) vs time curve for bending test of Mahan pecans.

han and Western Schley were more resistant than Barton and Wichita, but did not differentiate between Barton and Wichita or between Mahan and Western Schley.

A typical force vs time curve (Fig. 3) for bending showed a sharp drop in force followed by a steady increase until complete rupture occurred. The first and second peak were recorded including their force as well as distance from the start. The slope and area for the first peak were also recorded (Table 4).

CONCLUSIONS

COMPRESSION, TPA AND PUNCTURE can be considered suitable for measurements of texture of pecans. The variability of the readings and the agreement with sensory appraisals were similar for the three methods. Puncture had the advantage of being carried out directly on intact nut halves. Compression and TPA cylinders must be taken from the samples. Such preparation is time consuming and the cylinders must be handled with extreme care. However, TPA is not limited to one parameter but provides information about several properties.

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Ms received 2/4/94; revised 4/21/9; accepted 4/1/95.

Thanks are due to David Kidd of Cornell University (New York State Agricultural Experiment Station) for help in the preparation of the manuscript. We are grateful to the Head of the Faculty of Chemistry of the Universidad Autónoma de Chihuahua for material resources. We thank Boine Johnson (Texture Technologies Corp., Scarsdale, NY) for financial help.

The present investigation was part of the work for the M.Sc. thesis of co-author Araceli Ocón, who was assisted by a scholarship from CONACYT (National Board of Science and Technology), Mexico. Presented at the 54th Annual Meeting of the Inst. of Food Technologists, Atlanta, Georgia, 1994.

Inactivation of *Pseudomonas fluorescens* by High Voltage Electric Pulses

S.Y. HO, G.S. MITTAL, J.D. CROSS, and M.W. GRIFFITHS

ABSTRACT

A 30 kV pulsed power treatment system was designed and developed to process fluid materials. Our objective was to investigate the effects of high voltage electric pulses on microbial inactivation in an aqueous solution under different operating conditions and fluid properties. Electric field strength at 10 kV/cm for 10 pulses (2 sec pulse period and 2 μsec pulse width) with a spike of reverse polarity resulted in significant microbial control. *P. fluorescens* in various aqueous solutions were reduced in population by more than six log cycles. However, the critical electric field strength was affected by the nature of the pulse waveform across the treatment chamber which, in turn, was a function of electrode distance and fluid properties. Sudden charge reversal immediately at the end of a pulse provided maximum microbial decay.

Key Words: *P. fluorescens*, high voltage, electric pulses, pasteurization

INTRODUCTION

HEAT PASTEURIZATION is the most commonly used technique for food preservation (Knorr et al., 1994). Although heating inactivates enzymes and microorganisms, the sensory and nutritional properties of the food may decline because of protein denaturation and the loss of vitamins and volatile flavors. The quest for a nonthermal, energy efficient, and environmentally friendly pasteurization process has prompted rigorous research. The innovative technology of using pulsed power for food preservation appears to have potential benefits.

Many publications have demonstrated moderate to major microbial control in various aqueous solutions (Bushnell et al., 1993; Gupta and Murray, 1989; Sato et al., 1990, 1994; Jayaram et al., 1992; Castro et al., 1993; Knorr et al., 1994; Pothakamury et al., 1994; Whiting and Beacorn, 1994). Researchers have also demonstrated the possibility to process particulate or solid foods (Aibara et al., 1992; Geulen et al., 1993). However, the operating conditions and equipment varied to such an extent that results are hard to compare and generalize. An electroporation unit used for this research is unsuitable due to large pulse width, aluminum electrodes, very small sample size and limited controls for pulse manipulation.

Our objective was to determine the effectiveness of this innovative technique through a series of carefully designed experiments. A 30 kV pulsed power treatment system was designed and developed to process fluid materials of ≤ 148 mL batch volume. The batch unit could generate short duration pulses (2 μsec width) with electric field strength up to 100 kV/cm. The effects of these pulses on microbial inactivation in an aqueous solution were studied under different operating conditions (electric field strength, pulse period, and number of pulses applied) and different fluid properties (electrical conductivity, density, and rheological characteristics).

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MATERIALS & METHODS

Equipment development

A low cost pulse generator and treatment chamber were designed and developed. The pulsed power treatment system consisted of a 30 kV d.c. high voltage pulse generator and a circular treatment chamber (Fig. 1). The 110 V a.c. was raised in voltage through a high voltage transformer, and then rectified. The d.c. high voltage supply then charged up the 0.12 μF capacitor through a series of 6 MΩ resistors. The power source could provide up to 30 kV of high voltage. The resistors were immersed in oil to prevent corona and arcing. The generation of high voltage pulses relied on the discharge of the 0.12 μF capacitor through the thyratron. This produced a pulsed electrical field (exponential decay pulse) between the electrodes in the treatment chamber.

The pulse generator emitted a train of 5V pulses, and the trigger circuit served to convert that to 500V pulses using a silicon control rectifier (SCR). Terminologies for a pulse waveform are shown in Fig. 2. For our research, a typical pulse waveform was characterized by a pulse with 2 μsec width and 2 sec period (adjustable from 2 to 11 sec). In other cases, the pulse waveform is represented by a pulse and a spike (a low amplitude pulse with reverse polarity) with various widths.

The circular treatment chamber (25.0 cm diam) had two circular and parallel stainless steel electrodes (16.5 cm diam). The insulation (Delrin)

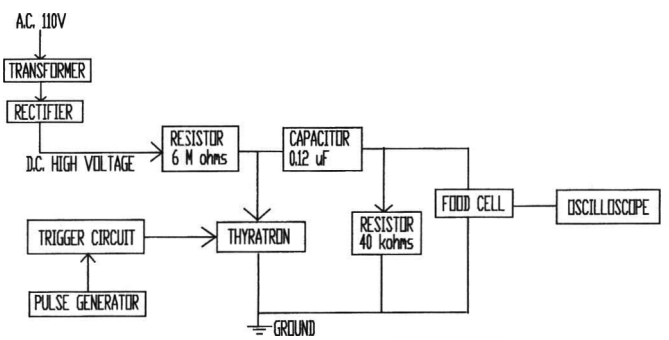

Fig. 1—Block diagram of pulsed power treatment system.

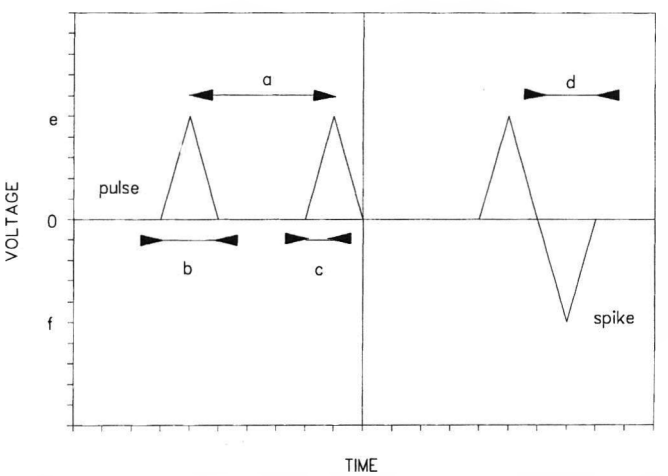

Fig. 2—Pulse waveform: profile and terminologies. a pulse period (sec); b pulse width (sec); c pulse rise time (sec) time to reach e (V); d spike width (sec); e pulse peak voltage (V); and f spike peak voltage (V).

Table 1—Selection of electrical parameters (Phase I) using horizontal chamber orientation

Electrode distance (cm)	Electric field strength (kV/cm)	No. of pulses applied	Pulse period (sec)
0.3	10, 15, 20, 25, 30, 35, 40, 45	10, 20, 30	2, 4
0.6	10, 15, 20, 25	10, 20, 30, 50, 100	2, 4
0.9	10, 15, 20	10, 20, 30, 50, 100	2, 4

Table 2—Selection of electrical conditions and fluid media (Phase II)

Parameters	Range
Electrode distance (cm)	0.3
Pulse period (s)	2
Pulse width (μs)	2
Number of pulses applied	10, 20
Electric field strength (kV/cm)	15, 25, 35
Chamber orientation	horizontal
Sucrose solution (% w/w):	Specific gravity:
10, 15, 20, 25, 30, 35	1.04, 1.06, 1.09, 1.11, 1.15, 1.16
NaCl solution (% w/w):	Electrical conductivity (μS/cm):
0.1, 0.2, 0.3, 0.4, 0.5	2400, 4200, 6150, 8100, 10200
Xanthan gum solution (% w/w):	Flow index:
0.2, 0.4, 0.6, 0.8, 1.0	0.82, 0.61, 0.41, 0.22, 0.16
	Consistency coefficient ($pa.s^n$):
	0.004, 0.014, 0.036, 0.076, 0.096

was constructed to have close physical contact with the electrodes. The distance between electrodes could be adjusted by inserting Delrin circular plates (14.5 cm diam) with thickness 0.3, 0.6, or 0.9 cm. Thus, the process volume could be varied between 49.5 mL, 99.1 mL, and 148.6 mL, respectively.

To eliminate air bubbles in the treatment chamber during electrical treatment, the sample could go into the cavity from the bottom through a channel in the chamber under 51 kPa vacuum. The cell was held in place on a hinge-jointed movable frame, so the sample could be treated either vertically or horizontally. All samples were treated using a horizontally positioned chamber to avoid electrical sparking. The use of a vertically oriented chamber could increase the possibility of gas pocket formation at the insulator/fluid interface by capillary action or gravity force, and thereby increase the chance of sparking as compared to a horizontal orientation. A digital storage oscilloscope was connected to the system so that each pulse across the chamber could be monitored.

Experimental methods

The study of microbial inactivation was divided into two phases. In Phase I, various electrical conditions were applied to a common fluid medium (0.1% peptone solution) containing *P. fluorescens,* a common food spoilage microorganism. In Phase II, a number of those electrical conditions were selected and applied to various fluid media containing the same type of microorganism. All tests in Phase II were performed using 0.3 cm electrode gap, 2 sec pulse period and horizontal chamber orientation. Three physical parameters were studied. Sodium chloride, sucrose, and xanthan gum were used to change the electrical conductivity, density, and rheological properties of distilled water respectively. Levels studied were selected to represent the normal ranges found in common liquid food products. Electrical conductivity was measured by using a conductivity meter (model L, Myron Corp., Carlsbad, CA), pH was measured using an Accumet pH meter (model 925, Fisher Scientific, Whitby, Ontario, Canada), temperature was measured using a temperature sensor (Digi-thermo, model 15-077, Fisher Scientific, Whitby, Ontario, Canada), and density by measuring mass and volume. Rheological properties were measured by using a wide gap rotational viscometer (model RVTD, Brookfield, Stoughton, MA). The method given by Rizvi and Mittal (1992) was used to measure viscosity of newtonian samples, and flow index and consistency coefficient for non-Newtonian samples (Tables 1, 2 and 3).

A pure *Pseudomonas* culture (M3/6, from Dept. of Microbiology, Univ. of Guelph, Guelph, Ontario, Canada) was prepared in Tryptone Soya Yeast Extract broth (Unipath, Hampshire, England, CM 802). The culture was kept at 25°C and adequate aeration was provided using a stirrer (Corning Glass Works, New York, Model PC-351). An inoculation period (48 hr) allowed sufficient growth and provided a concentration of around 10^8 cells/mL (early stationary phase). Stationary phase and concentration were measured by a spectrophotometer at 540 nm. For this microorganism, a calibrated growth curve at 540 nm was established and used to convert optical density to cell concentration. Microbial cells were harvested from the broth using a centrifuge (Beckman centrifuge, New York, Model J2-MC). Conditions were 5380g at 4°C for 10 min. Cells were then washed twice with sterile distilled water and resuspended in 0.1% (w/w) peptone solution (Phase I) or other designated solutions (Phase II).

Table 3—Fluid properties after sterilization at 121°C for 15 min

Solution	% (w/w)	Electrical conductivity (μS/cm)	Specific gravity	Rheological characteristics
Distilled water	100	1.10	1.00	1.0E-3 Pa.s
Peptone	0.1	650	1.00	1.0E-3 Pa.s
Sucrose	10	18.0	1.04	9.2E-3 Pa.s
	15	18.5	1.06	10.6E-3 Pa.s
	20	17.0	1.09	10.6E-3 Pa.s
	25	17.0	1.11	12.4E-3 Pa.s
	30	16.5	1.15	14.4E-3 Pa.s
	35	16.5	1.16	16.8E-3 Pa.s
Xanthan gum				n, m ($Pa.s^n$)[a]
	0.2	220	1.00	0.82, 0.004
	0.4	400	1.00	0.61, 0.014
	0.6	500	1.00	0.41, 0.036
	0.8	700	1.00	0.22, 0.076
	1.0	900	1.00	0.16, 0.096
Sodium Chloride	0.1	2400	1.00	1.0E-3 Pa.s
	0.2	4200	1.00	1.0E-3 Pa.s
	0.3	6150	1.00	1.0E-3 Pa.s
	0.4	8100	1.00	1.0E-3 Pa.s
	0.5	10200	1.00	1.0E-3 Pa.s

[a] n = flow index, m = consistency coefficient, $Pa.s^n$.

For each test, the sample at 20°C was fed into the treatment chamber from the bottom with a vacuum pump (Waters Millipore, Milford, MA, Model D0A-V152-AA) at 51 kPa to remove air bubbles. The vacuum was released after 30 s to eliminate any chance of sparking. High voltage pulse test was then performed on a horizontally oriented chamber and the pulse waveform was recorded using a digital storage oscilloscope and high voltage probe (Tektronix Inc, Beaverton, OR, Model 2230). The pulse treated sample was then kept at 4°C to minimize microbial growth. The treatment chamber and the sample feeding tubes were sanitized with chlorine bleach and rinsed thoroughly with sterile distilled water.

Before and after electrical treatment, plate counts indicated the reduction of microbial cells under different electrical conditions and suspending media, and were determined as follows: Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) were made for each test, and two samples of each dilution were prepared on agar dish (*Pseudomonas* agar base, Unipath, Hampshire, England, CM 559 and *Pseudomonas* CFC supplement, Unipath, Hampshire, England, SR 103) using a spiral plater (model D, Spiral System Inc., Cincinnati, OH). There was no need to use the selective medium which had been used in the beginning to isolate *P. fluorescens* in samples. However, CFC supplement isolated colonies from each other, thus it was easier to visualize individual colonies. Moreover, the same number of colonies were detected when CFC supplement was not used. Therefore, selective medium was used in all tests. All dishes were incubated at 25°C for 18 hr (18 to 36 hr were used initially, but in the course 18 h resulted in well developed colonies), and the microbial count for each dish was determined using standard spiral plating system procedures.

Calibration tests were carried out to indicate if there were any temperature increases, variations in physical properties, or dielectric breakdowns in the fluid samples. Control tests were also carried out to ensure that the microbial reduction obtained in the experiments was not due to the suspending medium (such as osmotic effect from the sucrose solutions) nor the bleach from sanitization. For all samples, HPLC analysis to measure sodium ions and pH analysis were also carried out.

RESULTS & DISCUSSION

Calibration tests

The degree of stability of the fluid media was tested under extreme electrical conditions: 27 kV at 0.3 cm electrode distance for 150 pulses at 2 sec period. The samples included distilled

water, peptone solution at 0.1%, sucrose solutions at 10 and 35%, xanthan gum solutions at 0.2 and 1.0%, and sodium chloride solutions at 0.1 and 0.5%. The physical properties in all solutions were unaffected. Knowing that the energy supplied per pulse to the chamber was 43.7J (0.5 × 0.12E-6 × 27E3²), the energy supplied per process mole in each pulse could be calculated to be 15.9 J/mol (process volume 49.5 mL). With a short pulse duration of 2 msec and a relatively long pulse period of 2 sec, this energy probably would not have enough time and intensity to cause any bond (intermolecular forces, covalent, ionic, etc.) breakages in the solutions.

A normal chemical bond has 418 kJ/mol energy, hydrogen bond has 17 to 25 kJ/mol energy, and weaker bonds like van der Waals forces have < 10 kJ/mol bond energy (Williams, 1987; Fox, 1991). Normally, sucrose only undergoes partial decomposition at 210°C and xanthan gum can maintain its stability at 80°C and under high shear. This probably explains the stable physical property readings throughout the experiments.

The temperature of the solutions also remained stable (20 ± 1°C at room temperature). Since the energy supplied per process volume in each pulse was only 0.88 J/cm³ whereas the heat capacity per process volume for water is 4.186 J/(cm³ · °C), this probably explains the steady temperature readings. Thus, pulsed power treatment, using the existing system, was proved to be nonthermal, and seemed not to cause dielectric breakdown nor changes in physical properties of the fluid medium.

Theoretically, although the typical formation of oxygen gas and hydrogen gas was possible, the amount would not be significant, especially when stainless steel electrodes were used, and the pulse width was so small. No gas formation was detected during the experiment, as the probability of sparking would have increased. Under careful examination, the electrode surface did not appear to have any abnormalities (e.g. pits). In addition, the energy required for electrolysis to occur was 241.8 kJ/mol at 1 bar and 25°C (Plzak et al., 1994) and the energy supplied per process mole in each pulse was calculated to be 15.9 J/mol. This amount would not be enough to trigger electrolytic reactions. Based on available information, the occurrence of electrolysis in any pulse treatments was not likely.

Effects of electrical parameters

Microbial reduction with respect to all tested electrical conditions was summarized (Table 4). Generally, the killing rate could be described as either "extremely high" or "drastically low." Knowing that the microorganisms were inoculated from a pure culture, the results confirmed that a critical potential had to be established on the cell membrane for electroporation to occur.

A pulse electric field strength of 10 kV/cm at 2 sec interval for 10 pulses was more than enough (Table 4) to achieve a microbial reduction of > 6 log cycles. At that combination, the voltage supplied to the chamber with 0.3 cm electrode separation was 4.5 kV, the processing time was 20 sec, and the energy and power supplied could be calculated to be 12J and 0.6W respectively. No significant effect of electrical field strength, pulse period and number of applied pulses on microbial inactivation was observed when 0.3 cm electrode gap was used. At wider gaps (0.6 and 0.9 cm), the microbial inactivations were very low even at 20 kV/cm and 100 pulses.

High temperature-short time (HTST) is a commonly employed thermal process for food pasteurization. The energy required to bring 49.5 mL of water from room temperature (20°C) to pasteurization temperature (71.7°C) can be calculated to be 10.7 kJ. Comparing that to the energy required (12J) to pulse treat the same amount of fluid using the current system, there is an obvious advantage (energy saving) in using pulsed power processing.

The critical field strength depended on the electrode distance. Due to the physical nature of the *Pseudomonas,* the chances of forming bacterial clusters in the peptone solution would not be high (Jay, 1992). The microorganisms should be evenly distributed in the space provided. Thus, the direct increase in electrode distance would not have much impact. Secondly, the number of pulses applied (≥ 10) and pulse period (≥ 2 sec) seemed not to be the deciding factors for microbial depletion in any of the electric field strengths being tested. Also, the controls in each test set indicated that inactivation was not due to osmotic effect nor sanitation. Thus, the differences in pulse waveform appeared to be the only reasonable element contributing to microbial reduction.

Under the same fluid medium and electrical conditions, a decrease in electrode distance to 0.3 cm gave rise to the formation of "spikes." The reversal of voltage might have caused an opposite dipole moment on the cell membrane, and at high repetitive rate, such action might have cut back the critical electric field strength required for electroporation. This explains the high killing rate when using the 0.3 cm electrode separation. Electric field strength required for significant microbial reduction was not established in the 0.6 and 0.9 cm electrode distances due to physical limitations of the treatment system. However, it was proven that field strengths of > 25 kV/cm for 0.6 cm separation and > 20 kV/cm for 0.9 cm were needed.

We hypothesized that the formation of spikes would increase the electric stress on the cell membrane. The application of bipolar pulses at moderate frequency should thus result in high microbial reduction at electric field strengths lower than expected. Knorr et al. (1994) applied 20 pulses to a suspension of *P. fluorescens* in 25 mL of UHT milk (1.5% fat) and obtained a microbial reduction of up to 4 log cycles. They reported that a critical field strength of 10.5 kV/cm and a critical treatment time of 20 μsec were necessary for the reduction. Results from our research seem to be in agreement.

Effects of fluid parameters

Microbial reduction with respect to all tested conditions (Table 5) showed as in Phase I, a high-or-low level of microbial reduction. This further confirmed that a critical electric field strength was required for microbial inactivation. Again, the number of pulses applied (≥ 10) seemed not to be the critical parameter. There was no change in microbial inactivation with increase in field strength from 15 to 35 kV/cm when sucrose and NaCl solutions were used. However, in xanthan gum solutions, microbial inactivations were very low at 15 kV/cm field, and very high at field strengths of 25 and 35 kV/cm. Since fluid properties were not the same, the reasons for those microbial reductions might be different.

With the sodium chloride solutions, the pulse waveforms had a similar profile as the peptone medium in Phase I. Due to the high conductance of the solutions, the spike electric field strengths were highest for the sodium chloride solutions. Sodium chloride solutions also had the highest spike to pulse duration ratio. The reversal of voltage and the resulting increase in electric stress on cell membranes were probably the cause of microbial reduction. The electrical conductivity test range in the sodium chloride solutions did not have a significant impact on the results (Table 5).

The pulse waveforms were the same in the case of xanthan gum solutions, a pulse followed by a spike. However, unlike the peptone and sodium chloride solutions, a higher field strength was required to cause significant microbial reduction. The xanthan gum might have created a protective shield for the microorganisms due to a high degree of interaction between polymer chains resulting in formation of a network of molecular aggregates held together by secondary valence forces. Thus, a higher electric field strength would be required to rupture the cell membrane.

The formation of spikes was a function of electrode distance and electrical conductivity (indicated by changing resistance and capacitance of the system). The sudden reversal of voltage was also hypothesized to be a principle cause for microbial reduc-

Table 4—Effects of electrical parameters on microbial inactivation

Electrode distance (cm)	Electrical field strength (kV/cm)	Pulse period (sec)	No. of pulses applied	Range	SV[a] Mean	sd[a]	N[a]
0.3	10, 15, 20, 25, 30. 35, 40, 45	2, 4	10, 20, 30	> 6.61 to > 7.29	> 6.96	0.21	48
0.6	10. 15, 20, 25	2, 4	10, 20, 30, 50, 100	0 to 0.56	0.24	0.18	32
0.9	10, 15, 20	2, 4	10, 20, 30, 50, 100	0 to 0.32	0.12	0.10	24

[a] SV = microbial reduction (log cycles) for the treatment; sd = standard deviation; N = number of experiments.

Table 5—Effects of fluid properties and electrical parameters on microbial inactivation

	Electric field strength (kV/cm)	15	15	25	25	35
	No. of pulses applied	10	20	10	20	10
				SV[a]		
Sucrose solutions	Range			> 6.06 to > 7.07		
at 10 to 35% (w/w)	Means/sd/N[a]			> 6.41/0.25/60		
NaCl solutions	Range			> 6.11 to > 6.64		
at 0.1 to 0.5% (w/w)	Mean/sdN			> 6.39/0.21/50		
Xanthan gum	Range	0 to 0.49		> 6.35 to > 6.65		
solutions at	Mean	0.13		> 6.5		
0.2 to 1.0% (w/w)	sd/N	0.14/20		0.11/30		

[a] SV = microbial reduction (log cycles); sd = standard deviation; N = number of experiments. Electrode gap = 0.3 cm, pulse period = 2 sec and horizontal chamber orientation were used for all tests.

tion. In the case of sucrose solutions, however, they exhibited minimal bipolar pulsing, and yet, substantial microbial reductions occurred. This may be due to a high osmotic pressure exerted by sucrose solutions.

Pulse dynamics

The pulse wave shape, pulse width and peak voltage across the treatment chamber appeared to be closely related to the electrode distance and electrical conductivity of the fluid. When a high voltage pulse of 10 kV was discharged to a fluid of electrical conductivity of 18 µS/cm (10% sucrose), the result was a typical positive pulse with 2 µsec width and the rise time was in the nanoseconds range. With a decrease in conductivity, the pulse width was increased. Thus, distilled water resulted in a positive pulse with about 20 µsec pulse width. With an increase in conductivity, the pulse width became smaller, and the resulting output was a positive pulse followed by a negative spike. The relative values of R, C, and L determined the relative size and shape of the pulse and spike. Results showed that the higher the electrical conductivity of the food, the shorter the duration of the pulse and longer the span of the spike. Also the spike to pulse peak voltage ratio increased as conductivity increased. With the increase in voltage at 0.3 cm electrode gap, there was a slight decrease of the spike to pulse peak voltage ratio in NaCl solutions, an increase in peptone solution, and no change in xanthan gum solutions.

Spike formation with charge reversal at the end of a pulse is completely different from a bipolar pulse. In the latter, polarity of the pulses is reversed alternately, as reported by Qin et al. (1994). At 16 kV/cm peak field and 18 µsec time constant of the exponential decay pulse, they reported more efficient inactivation of a *Bacillus subtilis* compared to monopolar pulses. They performed the experiments on an electroporator with an electrode gap of 0.1 cm and a very small volume of 100 µL. The polarity was reversed manually by rotating the cuvette 180° after each pulse. Thus, their results reflected the limitation of the equipment.

In spike formation, the charge reversal is sudden with no time lag, and an oscillating field is applied. The amplitude of the spike is not large enough to provide cell membrane breakdown, but they produce a high alternating stress on the cell membrane causing structural fatigue. Such mechanisms assist to damage the cell membrane in only a few pulses. This is the reason why microbial decay was not dependent mainly on pulse field strength or number of pulses. About 10 pulses were sufficient to provide desired microbial decay in liquids of various density, conductivity and rheological properties. These results will assist in developing an efficient and low cost commercial scale process and equipment compared to costly commercial pulsers.

CONCLUSIONS

ELECTRIC STRENGTH (> 25 kV/cm), number of pulses applied (≥ 10) and pulse period (≥ 2 sec) were not very important. Differences in output waveforms, which were affected by electrode distance, mainly led to microbial reduction. The critical field strength required for cell lysis was reduced by inducing additional stress to the cell membrane by bipolar pulses in sodium chloride solutions or the osmotic pressure in sucrose solutions. The critical electric field strength required for cell lysis could also be increased by creating a protective layer for the cells, as in the case of xanthan gum solutions. Studies on system behavior (modelling and response), ionic and particulate mobility, and cell membrane potential are needed to understand the nature of the pulse waveform with respect to fluid composition. Variations in microbial size, age and types should also be tested.

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High Pressure Effects on Emulsifying Behavior of Whey Protein Concentrate

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ABSTRACT

Oil-in-water emulsions (0.4 wt% protein, 20 vol% *n*-tetradecane, pH 7) prepared with solutions of pressure-treated (up to 800 MPa) whey protein concentrate (WPC) as emulsifier give a broader droplet-size distribution than emulsions made with native untreated protein. There was a decrease in emulsifying efficiency with increasing applied pressure and treatment time. In contrast, pressure treatment of corresponding WPC emulsions made with the native protein had little effect on emulsion stability. In the pressure-treated emulsion the protein is probably already conformationally modified so that pressure has little additional effect. However, in solution the native structure of the whey protein is modified by pressure resulting in loss of emulsifying efficiency.

Key Words: whey protein, emulsifier, high-pressure treatment, emulsion stabilization

INTRODUCTION

THE EFFECTS OF HIGH PRESSURE on food systems have been known for a long time. This technology is now being seriously considered as a method of food preservation and processing (Ledward, 1994; Galazka and Ledward, 1995). High pressure (i.e., up to 800 MPa) can modify protein structure and reactivity. Several reports (Zipp and Kauzmann, 1973; Balny and Masson, 1993; Defaye and Ledward, 1995; Defaye et al., 1995) have shown that pressure-induced dissociation and/or denaturation could be reversible under certain conditions. However, globular proteins may yield a modified conformation following treatment. Thus, the renatured protein may well have modified surface-functional properties when compared to the native protein. Pressure-treated ovalbumin and soy protein had improved emulsifying properties (Denda and Hayashi, 1992), but pressure treatment caused the foaming characteristics of egg white to be reduced (Knorr et al., 1992).

Whey protein concentrate (WPC) and whey protein isolate are produced in large amounts by the food industry as functional ingredients in many food applications (Morr, 1985; Morr and Foegeding, 1990). β-Lactoglobulin, the major whey protein, accounts for 55% of WPC (Morr, 1992). Some work has been reported on the influence of pressure on β-lactoglobulin in solution, and studies (Hayashi et al., 1987; Dumay et al., 1994; Hayakawa et al., 1994) have indicated that high pressure had a notable effect on its conformational and aggregation properties. Experiments have also shown (Galazka et al., 1995) that high-pressure treatment of pure β-lactoglobulin produced substantial loss of emulsifying efficiency.

Our objectives was to determine the effects of high pressure on properties of emulsions made with WPC. High-pressure treatments (200, 600 or 800 MPa) for various holding times (10, 20 or 40 min) were applied to the protein *before* homogenization, and also to the emulsion prepared separately with the native WPC. We wanted to determine whether high pressure processing was beneficial to functional properties of WPC. We also investigated the relationship between stability of WPC emulsions and the degree of adsorption of the protein emulsifier during pressure treatment.

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MATERIALS & METHODS

Reagents

Whey protein concentrate (containing 75% protein, 5% ash, 5% lactose, 9% fat, and 6% moisture) a food grade sample (Protarmor 75 P), was a gift from KWR Limited, Food Ingredients Division, (Basingstoke, Hampshire, England). Reagent grade *n*-tetradecane, buffer salts and sodium azide were purchased from Sigma Chemical Company (St. Louis, MO). Buffer solutions were prepared using deionized double-distilled water.

High-pressure treatment

Samples of protein solutions and emulsions (0.4 wt% WPC, 56 mL) were sealed in polyethylene bags and subjected to high-pressure treatment at 23 ± 2 °C (Galazka et al., 1995) using a prototype Stansted 'Food-Lab' high-pressure apparatus (Stansted Fluid Power Ltd., Stansted, Essex, U.K.).

Emulsion preparation

Emulsions of *n*-tetradecane-in-water (0.4 wt% WPC, 20 vol% oil, imidazole-HCl buffer containing 0.02M imidazole, adjusted to pH 7.0 with HCl) were prepared with both native and pressure-treated WPC using a small-scale homogenizer (mfc microfluidizer™ H-5000, Microfluidics Corporation, Newton, MA). After blending (Braun Multipractic Hand Blender MR 30) for 30 sec, the coarse emulsion premix was recirculated through the microfluidizer at 160 bar for 1 min at 25 °C. After preparation, and high-pressure treatment, sodium azide was added as bacteriocide so that its final concentration in the emulsion was 0.01 wt%. Emulsion samples were stored in a water-bath at 25 ± 1 °C, and droplet-size distributions were determined immediately and at regular intervals for premixed samples using a Malvern Mastersizer X Version 1.2a (Malvern Instruments Ltd., Malvern, U.K.) with a presentation code 2NHD (based on the refractive index (R.I.) of dispersant 1.3300 and particle R.I. 1.4564). Creaming behavior was monitored by visually measuring changes in thickness of cream and serum layers using a ruler when stored at 25 ± 1 °C in samples of 40 mm height.

RESULTS & DISCUSSION

DROPLET SIZE DISTRIBUTIONS of *n*-tetradecane-in-water emulsions (0.4 wt% protein, 20 vol% oil, pH 7) made with whey protein concentrate were compared (Fig. 1). The average droplet diameter of a fresh emulsion (Fig. 1a) made with untreated WPC was similar to the droplet-size distribution for the same emulsion (Fig. 1b) which had been subjected to high-pressure processing (800 MPa for 40 min). In contrast, (Fig. 1c) the emulsion made with pressure-treated WPC (800 MPa for 40 min) resulted in a significant increase in droplet size and distribution.

A useful and sensitive parameter for indicating differences in emulsifying capacity and emulsion stability is the average diameter $d_{43} = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$, where n_i is the number of droplets of diameter d_i (Dickinson and Galazka, 1991). The effect of high pressure on emulsions using pressure-treated WPC was shown in terms of this parameter (Fig. 2a) and extent of cream separation (Fig. 2b). Note that the average droplet diameter for native WPC oil-in-water emulsions was d_{43} = 2.05 ± 0.15 μm

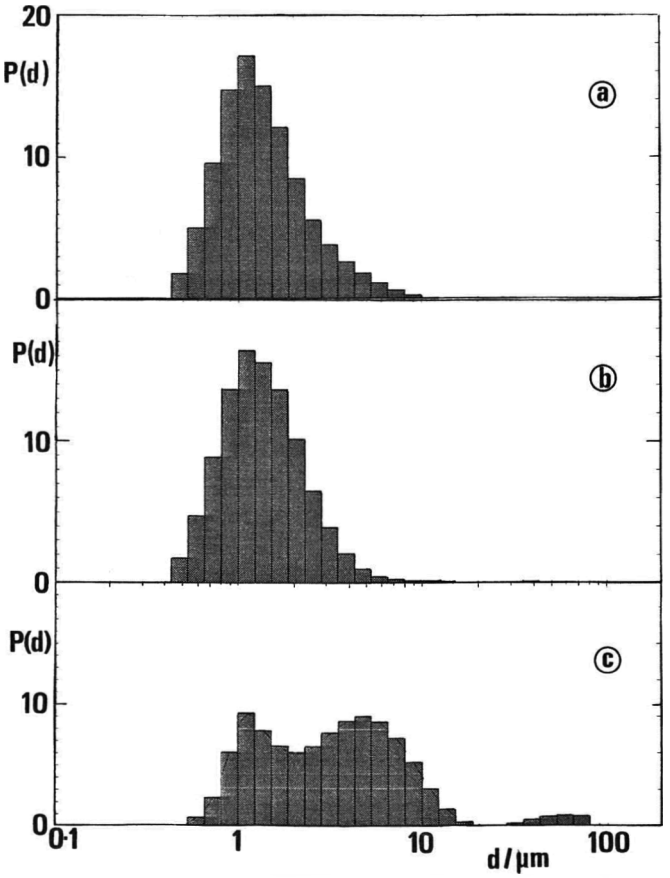

Fig. 1—Droplet size distributions of emulsions made with WPC. Probability function P (d) was related to droplet diameter d: (a) fresh emulsion without high-pressure treatment; (b) same emulsion after high-pressure treatment (800 MPa for 40 min); (c) emulsion prepared with pressure-treated protein (800 MPa for 40 min) before homogenization.

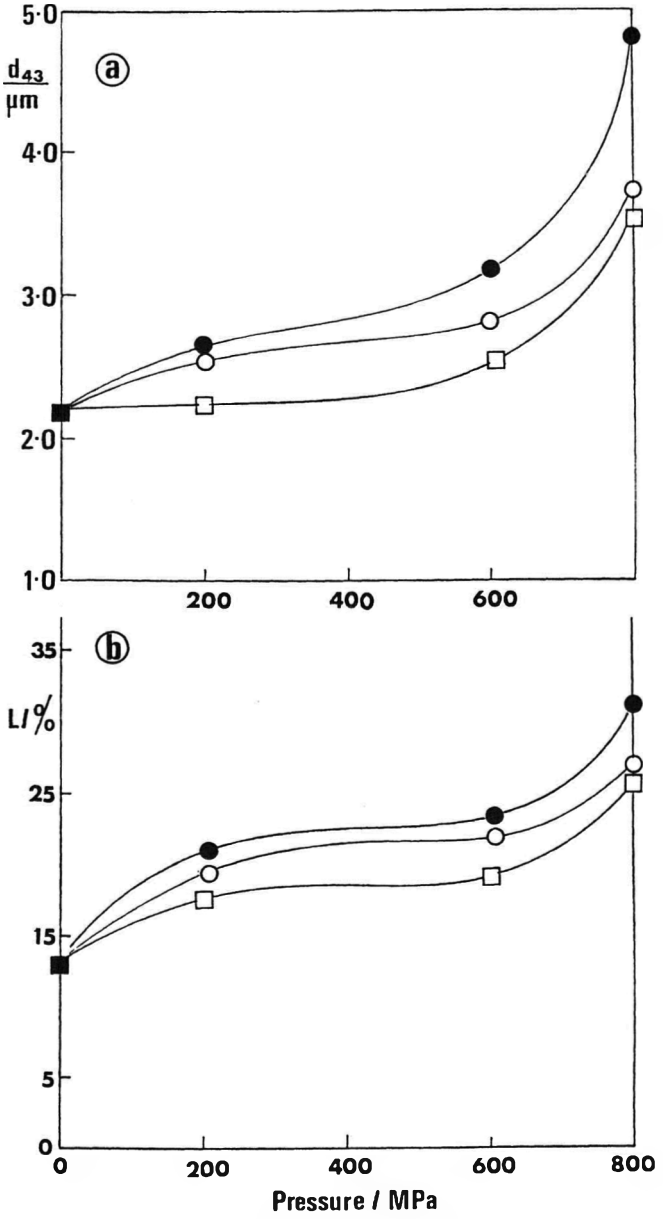

Fig. 2—Influence of high pressure on emulsifying efficiency of WPC pH 7.0. Oil-in-water emulsions were prepared using the mfc microfluidizer™ H-5000 at 160 bar and 25°C. Average droplet size d_{43} for fresh emulsions (a) and (b) the thickness, L, of the cream layer (expressed as percentage of total sample height) in 3-day-old emulsions was related to pressure and treatment times: no treatment (■); 10 min (□); 20 min (○); 40 min (●).

(mean of duplicates), and replacement of the untreated protein by a sample of pressure-treated WPC led in each case to emulsions with larger droplets.

The general trend was toward larger average droplet size with increasing pressure and treatment time. A treatment regime of 200 MPa for 20 min gave d_{43} = 2.57 ± 0.2 μm (mean and range of two determinations), whereas a more severe treatment of 800 MPa for 20 min gave an emulsion with d_{43} = 3.7 ± 0.44 μm (mean and range of duplicates). Over 3 days, in samples not subjected to agitation a distinct top cream layer, L, developed as determined by visual observation. There was a gradual increase in cream separation (Fig. 2b) with an increase in applied pressure and treatment time. However, the extent of creaming for the set of emulsions made with protein pre-treated at 800 MPa was much greater than for the control and other treatments.

The substantial change in emulsifying behavior of WPC, at pH 7, when subjected to high-pressure processing (200 MPa) was similar to that obtained with β-lactoglobulin (Galazka et al., 1995). This was expected since β-lactoglobulin is known to be the major functional protein in WPC. The combined results suggest therefore that the other components in WPC, including other globular proteins and lipid impurities, do not cause any qualitative change in effects of high-pressure treatment. Nevertheless, it is possible that high-pressure treatment has a different effect on structure of pure β-lactoglobulin protein than on possible β-lactoglobulin/lipid complexes in WPC. However, such changes in molecular structure might not necessarily manifest themselves in terms of measurable changes in emulsion properties.

We and others (Galazka et al., 1995; Dumay et al., 1994) have shown that β-lactoglobulin undergoes some limited aggregation following pressure treatment, and preliminary optical density data suggest some aggregation also occurs with WPC. This is presumably due to exposed hydrophobic regions and/or sulfhydryl groups of the whey proteins reacting to form oligomers. An increase in surface hydrophobicity (Hayakawa et al., 1994; Galazka et al., 1995) and loss of some secondary structure (Hayashi et al., 1987; Dumay et al., 1994) also occurs. However, aggregation of the whey protein is the likely cause of loss in functionality, since any increase in surface hydrophobicity should improve emulsifying efficiency (Nakai and Li-Chan, 1988).

High-pressure treatment of a WPC-stabilized oil-in-water emulsion made with untreated protein had little or no effect on droplet size (Fig. 1). During homogenization a protein probably becomes partially unfolded at the interface. Thus, the subsequent high-pressure processing causes no significant further confor-

mational change. High-pressure treatment of a whey protein emulsified oil-in-water system appears to have relatively little influence on the emulsion stability. However, the same treatment of the whey protein concentrate prior to emulsification could have a substantial negative effect. High-pressure treatment at ambient temperature, does not appear to improve emulsifying properties of whey proteins.

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This research was supported by the Ontario Ministry of Agriculture & Food through their Ontario Food Processing Research Program.

Rheological Properties of Renneted Reconstituted Milk Gels by Piezoelectric Viscoprocess: Effects of Temperature and Calcium Phosphate

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ABSTRACT

Scanning electron microscopy (SEM) and rheology and permeability were used to evaluate effects of clotting temperature and calcium phosphate on renneted reconstituted milk gels, especially microstructure and permeability. Dynamic measurements with a piezoelectric Viscoprocess determined rheological properties of curd after various times of renneting. An increase in clotting temperature accelerated specific rennet action and gel formation; maximum stiffness was reached faster but its value was weaker. Addition of calcium phosphate increased rate of gel formation. Maximum stiffness was reached faster but its value was not changed. Gel microstructure was not very different but its permeability coefficient decreased.

Key Words: coagulation, milk gels, SEM, permeability, rheology

INTRODUCTION

THE RHEOLOGICAL PROPERTIES of renneted reconstituted milk gels are important to curd syneresis and cheese composition. There have been numerous publications relating to rheological behavior (Marshall et al., 1982; Ramet and Weber, 1980; Storry and Ford, 1982; Walstra and van Vliet, 1986; Dejmek, 1987; Roefs and van Vliet, 1990) and microstructure of milk gel (Kalab and Harwalkar, 1973; Green et al., 1978; Brooker and Wells, 1984; Tarodo de la Fuente and Lablée, 1987; Guthy et al., 1989; van Vliet et al., 1989). However, the organization of the protein network and the effects of temperature on maximum curd firmness are not understood. Moreover, few studies included calcium phosphate addition which is common practice when making cheese from reconstituted milks.

We reported (Lagoueyte et al., 1994) by an original method of gel sample preparation for SEM observation, that renneted reconstituted milk gel was a porous semi-solid, with a casein network holding a large mass of liquid phase. It acted as a viscoelastic body due to its solid and liquid phases. Our objective was to use a new approach to study rheological properties of renneted milk. The rheometer we used consisted of a piezoelectrical ceramic probe, and was not only a laboratory instrument but could be used also in pilot plant cheesemaking. It indicated the firmness of curd in physical units ($N \cdot m^{-2}$). Moreover, it probably would allow accurate on-line determinations of optimum coagulum firmness for cutting which is needed to achieve optimum cheese yield and cheese. We also carried out a rheological study of renneted reconstituted milk gels when clotting temperature and calcium phosphate concentrations were changed in relation to microstructure and permeability of the curd.

MATERIALS & METHODS

Milk

Reconstituted skim milk was made by dissolving a low-heat powder (Lait Matines-SILL, Plouvien, France) at 12% (w/v) in deionized water and storing it at 4°C for 12 hr.

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Calcium was added at 436 mg/L as $CaCl_2 \cdot 2H_2O$ and the milk was used in all experiments studying temperature effect (Table 1).

Calcium was added at 2.7421 g/L as $Ca(H_2PO_4)_2 \cdot H_2O$ and the milk was used in all experiments studying calcium phosphate effects. This milk had the same concentration of calcium as the other one but a different concentration of phosphate. All experiments were conducted at 32° ± 0.1°C. To prevent bacterial growth, 0.1% (w/v) sodium azide was added. Before rennet addition, reconstituted skim milk was heated at 20°C for 2 hr, and subsequently at each temperature for 30 min. After 2 hr at 20°C, the pH of the milk was measured. It was different following addition of salts because no corrections were made: pH was 6.24 for the first milk and 5.94 for the second.

Rennet clotting time (RCT) and curd cutting time (CT)

Commercial rennet (520 mg chymosin/L, Sochal, St Etienne de Chaumeil, France) was used at 25×10^{-3}% (v/v) and changed when loss of activity reached 1%. Rennet clotting time (RCT) was recorded from rennet addition to the onset of aggregation by the method of Sommer and Matsen (1935). Curd cutting time (CT) was the time elapsed from rennet addition to the moment when gel firmness reached a predetermined level, i.e., the firmness obtained after 300 min at 26°C (Lagoueyte et al., 1994) with calcium-enriched milk.

Penetrometry

Gel firmness was determined by penetrometry. The apparatus used (PNR 10, Surbelin-Sommer and Runge KG, Berlin, Germany) was fitted with a conical probe (Ref.18.2361), so its penetration was in inverse ratio to firmness (Lagoueyte et al., 1994).

Rheological measurements

Dynamic measurements were performed with the rheometer "Viscoprocess" (METRAVIB, Ecully, France) consisting of a probe, an electronic part for signal transmission and a computer for data processing. The probe consisted of two piezoelectrical ceramics (Merzeau, 1985), arranged face to face (Fig. 1a). When an electrical tension was applied to one ceramic, its dimensions were changed: the sinusoidal electrical signal was transformed into mechanical deformation. Conversely, the piezoelectrical ceramic transformed mechanical deformations into electrical signals. If a sample (fluid, solid or viscoelastic material) was confined between two piezoelectrical ceramics, it transmitted the deformation from one to the other.

For all experiments, the probe was inserted into a thermostated beaker filled with 400 mL milk held at desired temperatures (26°, 32° or 40 ± 0.1°C) (Fig. 1b). After rennet addition, the milk was overlaid with a thin layer of paraffin oil to prevent drying at the surface. Measurements were started 1 min after renneting. In aging experiments, the properties of gels were measured during 24 hr at a frequency of 10 Hz. The stiffness K and tan δ were automatically recorded by the Viscoprocess. To evaluate the RCT by the Viscoprocess apparatus, we considered the point where the stiffness became greater than the initial mean value calculated be-

Table 1—Composition of reconstituted skim milk (% w/w)[a]

Dry matter	Ca	Phosphorus	Lactose	Acidity (lactic acid)	Total nitrogen	NPN	Fat
10.503 ±0.010	0.151 ±0.004	0.106 ±0.002	5.63 ±0.20	0.273 ±0.004	3.93 ±0.05	0.20 ±0.02	trace —

[a] Mean value of three experiments with standard deviation.

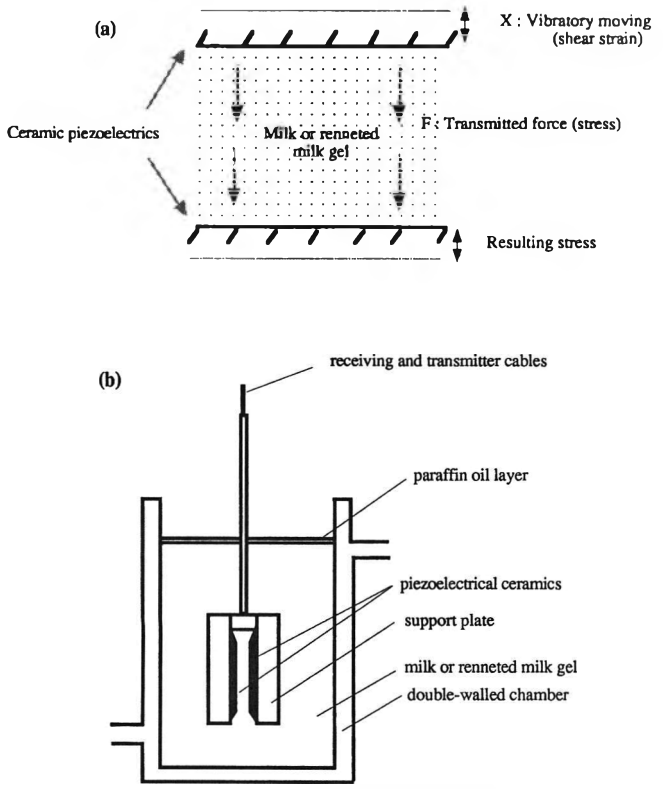

Fig. 1—Viscoprocess probe. (a) principle; (b) schematic representation.

tween the 1 and 3 min after renneting. We called this time the gel point (Fig. 2a).

Moreover, RCT was determined concurrently with gel firmness, gel permeability and microstructure analysis.

Scanning electron microscopy (SEM)

The method for SEM was as described before (Lagoueyte et al., 1994). A prism-shaped parallelepipedic probe (~3 mm^3, 0.1 to 0.5 mm thick), open on three sides, was made by folding a thin copper sheet (0.15 mm thick). This was inserted into the milk 30 min before rennet addition to allow for temperature equilibration. After CT, the probe with its enclosed piece of gel (0.1 to 0.5 mm thick) was removed and rapidly inserted into Freon 22 which was cooled and kept at −155°C by liquid nitrogen (at −196°C) to provide a high freezing rate and limited ice crystal growth, in the part of sample closest to the copper sheet (Robards and Sleytr, 1985). The rapidly frozen gels were subsequently freeze-dried (Alpha 1–4; Martin Christ, Osterode, Germany) with the cold-trap at ≤−80°C. SEM observations were made with a Leica Stereoscan 260 (Leica, Villepinte, France).

Permeability

Gel permeability coefficient (B) was determined by the technique of van Dijk and Walstra (1986). Perfectly clean, 3-mm-internal-diameter glass tubes were inserted into renneted milk prior to clotting. Under such conditions, the gel did not shrink and showed no syneresis. After CT, the tubes were withdrawn and transferred to a whey bath and submerged so that resulting hydrostatic pressure (ΔP) caused the whey to permeate upward through the gel in the tube:

$$\Delta P = \rho whey \cdot g \cdot h_0 \text{ at a level } h_0 \text{ below whey surface (expressed in m of gel:kPa·m}^{-1})$$

The rate of permeation of whey was used as a measure of (B) (van Dijk, 1982). Two resulting hydrostatic pressure values were used assure that permeability was independent of pressure.

RESULTS & DISCUSSION

Validity of dynamic measurements

The absolute values of stiffness and of tan δ of renneted reconstituted skim milk enriched with 436 mg/L of calcium increased with oscillation frequency in the range 10 to 50 Hz. However, the frequency increase implied greater background noise, with the maximum at 30 Hz. Consequently, we used a frequency of 10 Hz with a minimal background noise.

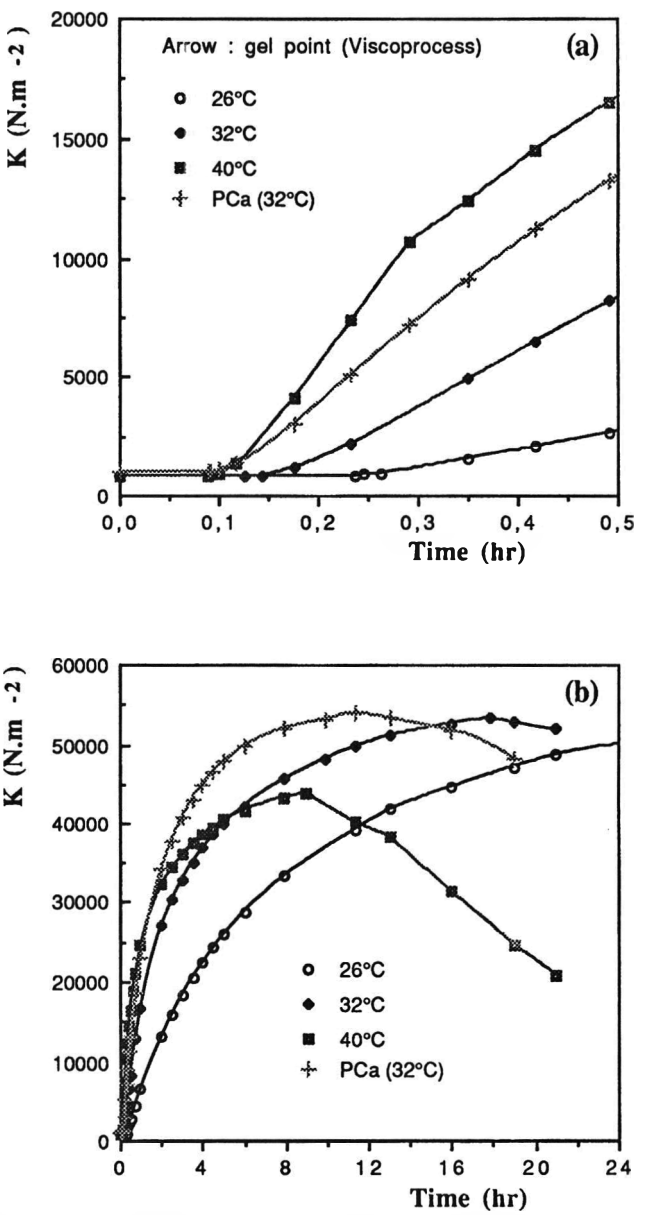

Fig. 2—Effect of temperature clotting and calcium phosphate on the stiffness of curd. (a) during the first 30 min after renneting; (b) during 24 hr after renneting.

In order to validate this method, we compared the stiffness with RCT (Sommer and Matsen, 1935) and gel firmness (penetrometry). The gel point was that point of gel transition when its detection appeared shortly after RCT, as determined when the visible flecks were seen in a rotating test tube. The gel point was in agreement with RCT, so it indicated reliability of the results (Table 2). Moreover, the ascending part of the stiffness curve characterized gel firming and was similar to that of penetrometry (Fig. 3). This indicated that stiffness and firmness were related.

Effect of clotting temperature

Results for K were recorded during the first 30 min after renneting (Fig. 2a) and after 24 hr (Fig. 2b). A sigmoid-shaped curve of K vs time resulted. After a constant stage, stiffness increased very fast, followed by a slower increase until a maximum value was reached and subsequently, K decreased. The

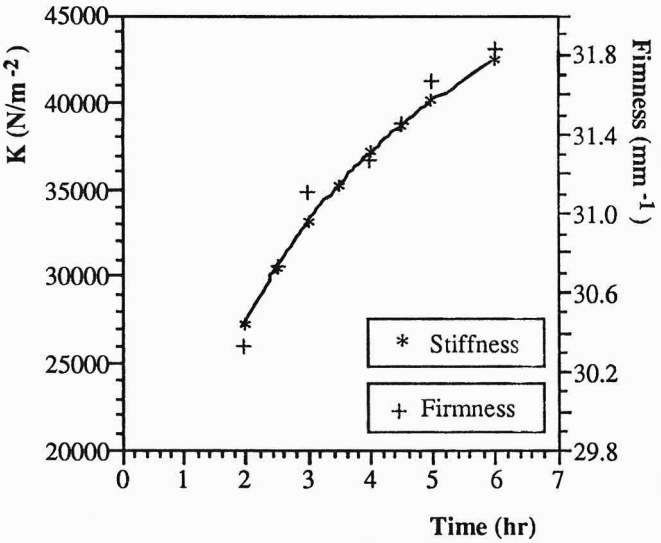

Fig. 3—Kinetics of stiffness and firmness between the 2nd and the 6th hour after renneting.

Table 2—Effect of temperature and calcium phosphate addition on rennet clotting time, curd cutting time and on characteristic values of K and tan δ

	26°C	32°C	40°C	Calcium phosphate addition (T=32°C)
Rennet clotting time[a] (min)	12.72 ± 0.23	8.30 ± 0.16	5.33 ± 0.12	6.18 ± 0.07
Cutting time[b] (min)	300 ± 9	162 ± 10	239 ± 16	167 ± 13
Initial value[c] of K ($N \cdot m^{-1}$)	832 ± 12	818 ± 5	808 ± 13	985 ± 42
Gel point[c] (min)	14.25 ± 0.45	8.72 ± 0.35	5.63 ± 0.38	7.03 ± 0.12
Initial value of tan δ[c]	8.6 ± 1.7	5.8 ± 1.2	5.2 ± 1.0	36.8 ± 5.6
Lag time of tan δ (min)[c]	10.00	5.00	4.00	5.00
60% of gelation time (min)	7.63	4.98	3.2	3.72
Maximum value of tan δ[c]	16,4 ± 0.3	16,6 ± 0.5	16,2 ± 0.2	

[a] Mean values and standard deviation of RCT determined by the method of Sommer and Matsen (1935). Total samples: p = 10 at temperatures 26°C and 40°C and for calcium phosphate addition, p = 12 at 32°C.

[b] Mean values and standard deviation of CT. Time to obtain gel firmness equal to that at 300 min at 26°C. See Materials & Methods. Total samples: p = 5 at temperatures 26°C and 40°C and for calcium phosphate addition, p = 6 at 32°C.

[c] Mean values and standard deviation determined by the Viscoprocess. Total samples p = 5 at temperatures 26°C and 32°C, p = 6 for calcium phosphate addition, p = 7 at temperature 40°C.

start of the stiffness increase agreed with RCT as determined by the method of Sommer and Matsen (1935) (Table 2) and K varied with time as evidenced by gel firmness measured by penetrometry (Fig. 3).

Initial stiffness was not different when temperature was raised (Table 2). After 1 hr, the stiffness was 2.7 times higher at 32°C and about fourfold greater at 40°C than at 26°C (Fig. 2b). The rate of strengthening of the gel directly after onset of clotting also increased with temperature, but the maximum firmness, reached at 40°C, was lower than that at 32 or at 26°C. At 26°C, K increased slowly with time and after 24 hr it was still increasing (K was greater than 55,000 $N \cdot m^{-2}$ after 48 hr). At 32°C, the modulus reached maximum after 18 hr and then decreased slowly. At 40°C, the maximum, as well as the subsequent decrease of modulus, was reached sooner (9 hr) than at 32°C. The maximum decreased with increasing temperature. The observed decrease in plateau value of the modulus with increasing temperature was similar to results of Roefs (1986) and Zoon et al. (1988b).

The decrease in stiffness maximum value with increasing temperature was unexpected because the hydrophobic interaction (major interaction forces in clotting rennet milk) was expected to strongly increase. The increase in number of intermicellar bonds and of the rate of bonding with temperature led to a faster rate of firmness, but, concurrently, the rapid increase of bonding also caused faster syneresis. Therefore, the curve representing stiffness kinetics could be related to the fact that the increase in coagulum firmness was quickly masked by syneresis, because the sample placed between the two ceramics became nonhomogeneous and K values decreased.

Kinetics of tan δ during the first hr (Fig. 4a) after renneting and after 24 hr (Fig. 4b) were compared. For each temperature, a lag phase followed by a very fast increase to a maximum was observed (when K remained constant). Afterwards it slowly decreased to a minimum value. At 40°C, tan δ increased again after 8 hr of renneting.

As tan δ is the ratio of sin δ to cos δ, i.e., the ratio between viscous and elastic components, an increase in tan δ indicated that viscous character increased and elastic character decreased. This implied that viscous character was prevailing. In the same way, a decrease of tan δ indicated that elastic character became prominent.

Lag time and initial value of tan δ decreased with an increase in temperature (Table 2). Tan δ started to increase before gel point, indicating the viscous character of the aqueous phase and the time to obtain its maximum value varied with temperature (40 min at 26°C, 17 min at 32°C and 9.5 min at 40°C). The maximum value of tan δ was not significantly affected (Table 2). At 26°C the increase of tan δ occurred when more than 80% of RCT had passed, whereas at 32 or 40°C this trend occurred after only 60% of RCT occurred. Since milk was still liquid, this was perhaps due to the increase of the rate of para-casein micelle aggregation: the formation of larger particles increased the viscosity of the aqueous phase. This agreed with the hypothesis of Green et al. (1978) and of van Hooydonk et al. (1986) that the secondary phase of rennet clotting starts before the end of the primary phase, when 60% to 70% of RCT has elapsed.

The decrease of lag time and of the initial value of tan δ have not been described with dynamic measurements because the studies of van Dijk (1982), Roefs (1986), Dejmek (1987), Zoon et al. (1988a) started only when elastic modulus (G') had reached a certain value (2 $N \cdot m^{-2}$), in order not to disturb gel formation, for which tan δ was already decreasing. However, we can note that the evolution of tan δ may also be related to the loss of viscosity after milk renneting at the natural pH of milk (pH = 6.7–6.8) (Scott-Blair and Oosthuizen, 1961; van Hooydonk et al., 1986; Sharma et al., 1989). This corresponds to the decrease in casein micelle volume resulting from enzymic reactions. Nevertheless, at the pH = 6.20 this result was not observed by van Hooydonk et al. (1986). They assumed that, at that pH and with the experimental conditions used, the aggregation of casein micelles compensated for the decrease in voluminosity.

When tan δ started to decrease, the elastic character became more prominent and indicated sol-gel transition. These results were in agreement with the conclusions of Bohlin et al. (1984) showing that the increase of the elastic modulus and the decrease of the tan δ occurred with the second phase of rennet clotting. The decrease of tan δ after the gel point to the minimum, concurrently with the increase of K, showed that the elastic component increased accordingly with gel formation. Tan δ reached the minimum faster and the value of the minimum was greater with increasing temperature (Fig. 4b), probably because network structuring was faster.

After 9 hr of clotting, tan δ increased slowly again at 32°C. This second time increase was much faster at 40°C (after ≈ 3 hr), whereas it was absent at 26°C, even after 48 hr clotting (tan δ was about 14). As Zoon et al. (1988b) reported, tan δ at 26°C should be lower than at 32°C. We suggest that, since the aqueous phase was purely viscous, the increase of tan δ could be related to the circulation of the aqueous phase, i.e., with the porosity

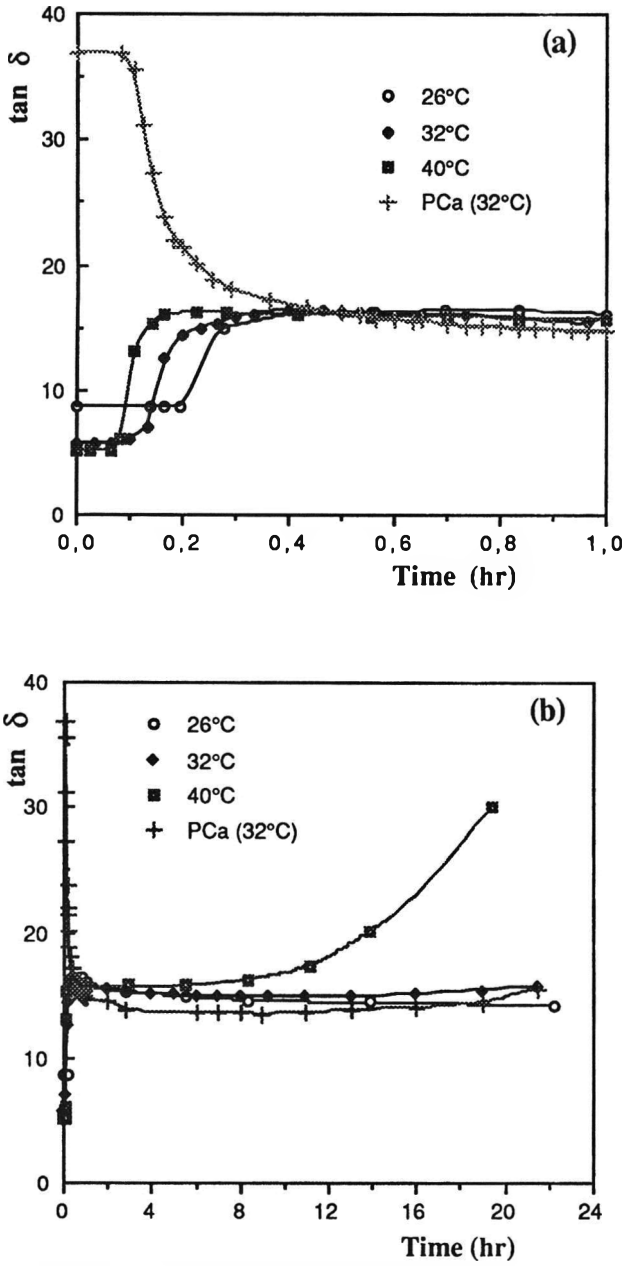

Fig. 4—Effect of temperature clotting and calcium phosphate on tan δ. (a) during the first hour after renneting; (b) during 24 hr after renneting.

and permeability of the gel. The increase of tan δ after gel formation could be due to the contraction in the gel network and its increase in permeability. This indicates that, after gel formation, the increase in network rigidity promoted a better aqueous phase circulation inside the network and, consequently, an increase in continuity of the aqueous phase and in gel permeability. The rigidity of the network would promote the transfer of the transmitted force by the piezoelectrical ceramic, but the chain breakdown due to network contraction would decrease it. When tan δ increased, the viscous component became more important compared to the elastic component. This should be considered as related to syneresis, since when the clotting temperature increased, tan δ increased after gel formation with simultaneous moisture expulsion.

Our results confirmed that the enzymatic reaction rate increased and the RCT decreased with increase in temperature which, in turn, also increased the gel strength due to fusion of micelles at a faster rate (Tarodo de la Fuente and Frentz, 1966; Cheryan et al., 1974; Ramet and Weber, 1980; Storry and Ford, 1982; Marshall et al., 1982; Sharma et al., 1989). Initially this

Table 3—Effect of calcium phosphate on permeability[a] of curd at 32°C

Calcium phosphate	ΔP kPam^{-1}	Samples	B×10^{-13} m^2	sd	B*×10^{-13}[b] m^2	sd
without	834	5	1.51	0.04	1.53	0.04
	540	3	1.55	0.02		
with	834	5	1.30	0.03	1.27	0.05
	540	3	1.22	0.03		

[a] Measured on coagulum at CT.
[b] B* = average permeability coefficient.

caused a higher modulus, because more bonds were formed, but the network formation was achieved earlier, so maximum firmness was reached sooner (Zoon et al., 1988b). The decrease in maximum firmness with increase of temperature was also reported by Tarodo de la Fuente et al. (1969), Ramet and Weber (1980) and Roefs (1986). According to van den Bijgaart (1988), with increasing temperature, the rate of change in permeability with time was higher and the maximum in endogenous syneresis pressure was reached sooner after rennet addition. These factors imply a more rapid coarsening of the network and would thus result in lower maximum firmness. Hydrophobic bonding and Brownian motion increase with increasing temperature. Consequently, the number of breaking protein strands are more important. Walstra and van Vliet (1986) suggested that, with increasing temperature, the rearrangement of the caseinate network leads to local condensation of the network, causing wider pores. This was described (Lagoueyte et al., 1994), as related to the effects of clotting temperature on microstructure and on the permeability coefficient. Since the permeability coefficient increased with clotting temperature, and based on the hypothesis of Walstra and van Vliet (1986), we assumed that the pores would interact with one another leading to better permeability.

Effect of calcium phosphate

Results (Table 2) showed that RCT was shorter for milk enriched with calcium phosphate, whereas cutting time was not affected. Phosphate addition at constant pH promoted the formation of micellar aggregates because the phosphate would favor calcium binding to micelles, and this would alter the net charge of caseinate aggregates (Creamer and Yamashita, 1976). A decrease in pH would lead to solubilization of many micellar components (Shalabi and Fox, 1982). The decrease in RCT was also probably due to pH since the optimum pH of rennet action was around pH 6.0 (van Hooydonk et al., 1986). Probably both the voluminosity of casein micelles was at a minimum and the accessibility of the Phe-Met bond of κ-casein was increased because of the diminished steric hindrance caused by the casein macropeptide released from the κ-casein.

Results for K (Fig. 2a and b) indicated no difference in stiffness kinetics between milks with or without calcium phosphate addition. The resulting action of calcium phosphate addition and of concomitant pH reduction led to shorter gel points and faster rates of gel formation (Table 2) (Fig 2b). Maximum firmness was reached faster, but the value was the same as with renneted reconstituted milk without addition of calcium phosphate.

Changes of tan δ were in the opposite direction whether the milk was enriched or not with calcium phosphate (Fig. 4a and b) (Table 2). Without calcium phosphate addition, the increase of tan δ before RCT indicated that associations between micelles led to more viscous body than in the initial milk. When calcium phosphate was added, its fixation on micelles or submicelles led to new organization characterized by a more intersubmicellar or intermicellar bonding resulting in an increase of the initial tan δ. The decrease of tan δ with gel formation agreed with an increase of gel elastic component. However, the final values of K (Fig. 2b) and tan δ (Fig. 4b) were the same with or without calcium phosphate addition. Consequently, calcium phosphate addition had no effect on maximum gel firmness value before

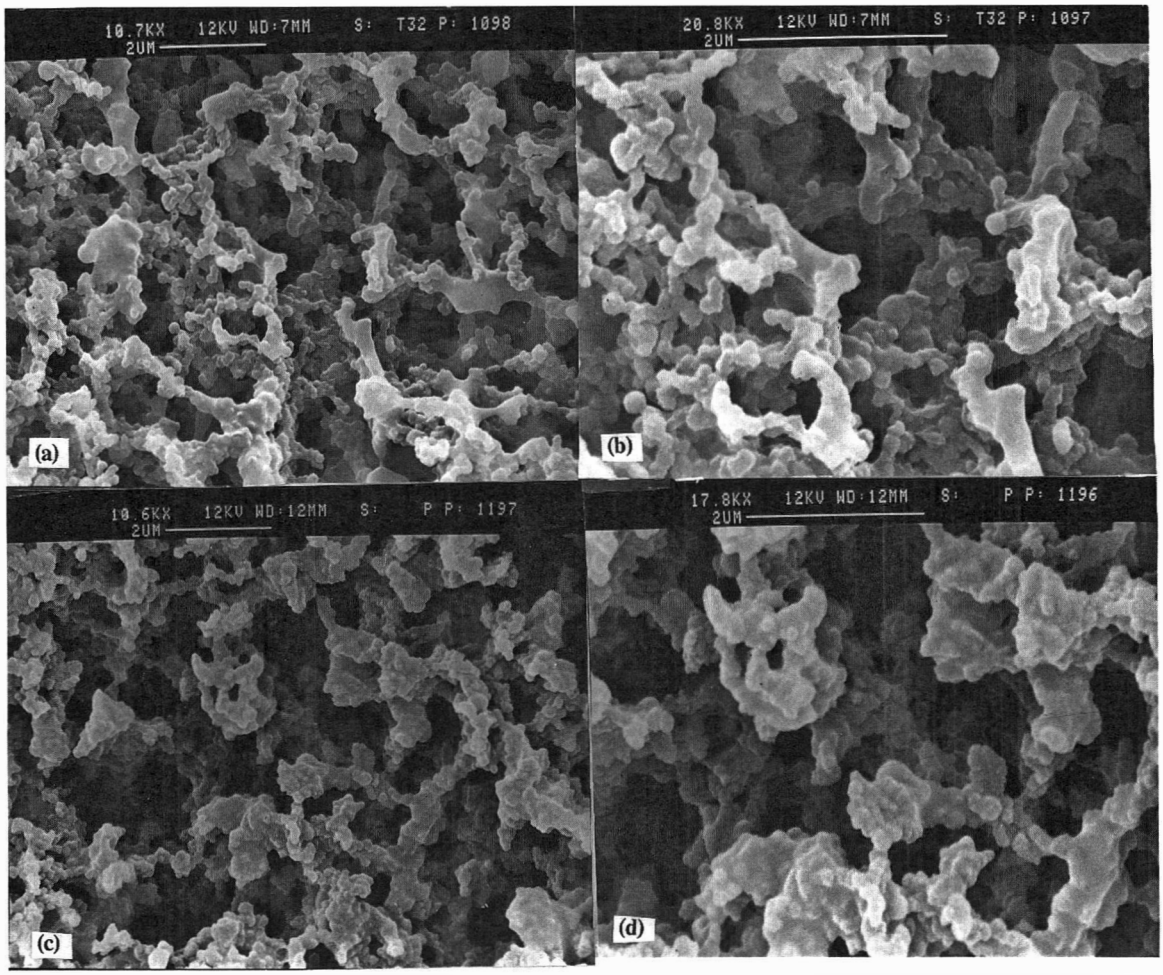

Fig. 5—Scanning electron micrographs of rennet-reconstituted milk gel. Top: After 162 min at 32°C without calcium phosphate addition: [(a) X10000; (b) X20000]; Bottom: After 167 min at 32°C with calcium phosphate addition: [(c) X10000; (d) X20000].

syneresis. The minimum value of tan δ was reached when 70% of maximum stiffness time elapsed (50% in the case of milk without calcium phosphate addition). Then tan δ very slowly increased after 8 hr (9 hr for milk without calcium phosphate addition). We assumed that the increase in network rigidity promoted a better aqueous phase circulation inside the networking and, consequently, an increase in continuity of the aqueous phase. Thus, tan δ changed very little because the aqueous phase variation favored the viscous component which compensated for the increase in network rigidity.

The permeability of milk with added calcium phosphate (Table 3) was less than that of the control. This suggested that the phosphate promoted intermicellar bonding, leading to a denser network with more continuous walls and, thus, retarded of serum flow. Nevertheless, the pH reduction favored serum circulation within the network, since, when the pH decreased from 6.3 to 6.0, permeability increased from $2.10^{-13}m^2$ to $3.10^{-13}m^2$ (van Dijk, 1982). Therefore, the low permeability of milk enriched with calcium phosphate could be offset by reduction of pH.

Scanning electron microscopy

To avoid artifact formation, which might arise from slow ice crystallization, we used samples 100 to 500 μm thick and observed structures immediately below the surface after sample fracturing. We observed 2 samples of 4 different gels in various directions. Under these conditions, up to a depth of about 10–20 μm, the microstructure was not distorted by excessively large ice crystal development (Robards and Sleytr, 1985).

The coagulum microstructures at CT by SEM analyses (Fig. 5) showed gels with or without calcium phosphate addition were not very different. However, the protein network had greater gaps, the micellar strands seemed to be denser, and the casein micelles also appeared to be fused together more tightly when milk was enriched with calcium phosphate. These observations confirmed the previous hypothesis that phosphates favored bonding.

CONCLUSION

THE VISCOPROCESS enabled us to follow the process of milk clotting up to syneresis without disturbing the system. The weak deformations imposed by the Viscoprocess reduced considerably the risk of gel breaking, which was not possible with the conventional viscosimeter. This apparatus was easy to use in the laboratory and in the cheesemaking plant and it provided objective indications of firmness and the viscous or elastic character of the gel. Addition of calcium phosphate seemed to lead to the same changes as obtained with increasing temperature. The great extramicellar porosity was more important than the small extramicellar porosity. The stiffness of the network walls, which were denser when calcium phosphate was added to the milk, explained the lower permeability coefficient.

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Casein Hydrolysate Produced Using a Formed-in-Place Membrane Reactor

WEN-DEE CHIANG, CHRISTOPHER T. CORDLE, and RONALD L. THOMAS

ABSTRACT

Casein hydrolysate was continuously produced by hydrolysis of bovine casein with Protease Type XXIII (from *Aspergillus oryzae*) in a pilot-scale formed-in-place membrane reactor. A high percentage (>99%) of TCA soluble nitrogen in the hydrolysate (product) was achieved after 45 min at 37°C and pH 7. The product was completely soluble over pH range 2–9. Water sorption increased 4 to 6.5 times at water activity of 0.35–0.95 as compared to intact casein. The immunologically active casein and immunologically active whey protein in the product were reduced 99% and 97%, respectively. Long term operation showed that the membrane reactor maintained steady production of casein hydrolysate longer than 17 hr.

Key Words: casein hydrolysate, protease, membrane reactor, immunological tests

INTRODUCTION

PROTEIN HYDROLYSATE with known functional and immunological properties can be widely used in food systems. Uses include improving food texture by increasing water-binding capacity, fortifying acidic beverages with a wide range of pH solubility, and developing infant formula with reduced allergenicity.

Production of protein hydrolysates enzymatically can be done by conventional batch hydrolysis or continuous hydrolysis using an ultrafiltration (UF) membrane reactor. Disadvantages of a conventional batch process include relatively high cost of enzymes which are only used once (Cheftel et al., 1971), low productivity (Prendergast, 1974), inconsistent quality, high labor cost and high energy requirements. In contrast, Iacobucci et al. (1974) developed a constant flux membrane reactor for producing hydrolysates. They concluded that the productivity of the reactor was 100-fold greater than batch hydrolysis. Mannheim and Cheryan (1990) also indicated that continuous processing with UF membrane systems allowed reuse of enzymes, thus reducing cost of enzymes and resulting in higher productivity. Furthermore, it is difficult to control the extent of reaction in batch processes, resulting in nonhomogeneous products of varying molecular weights (Cheryan, 1986). A UF membrane reactor has the potential to provide a consistently uniform product with desired molecular weight characteristics (Deeslie and Cheryan, 1981).

Formed-in-place (FIP) membranes are made by deposition of either inorganic solutes or organic polymers within the matrix of a porous tube (Marcinkowsky et al., 1966). The tube may be made from a variety of porous materials, including carbon, ceramic, or stainless steel. Processes for using FIP membranes in the food industry have been described by Thomas and Barefoot (1988). One advantage of using FIP membranes over cast membranes is easy removal and reformation of the membrane (Spencer and Thomas, 1991). Under severe fouling conditions, rejuvenation of FIP membranes by removal and reformation was much more economical than replacing the module with cast membranes. Another advantage is potential to form a wide variety of membranes with highly selective permeability. FIP membranes have been successfully used for production of apple juice (Thomas et al., 1986), and glucose (Wang, 1991) and selective enrichment of IgG from whey protein (Thomas et al., 1992).

Our objectives were to evaluate the performance of a continuous formed-in-place membrane reactor for production of casein hydrolysate by measuring its productivity and stability and to determine product quality and consistency by measuring functional and immunological properties.

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MATERIALS & METHODS

Substrate and proteolytic enzymes

Casein from bovine milk containing 90% protein (biuret method) and 0.5% lactose and Protease Type XXIII (from *Aspergillus oryzae*) were purchased form Sigma Chemical Co. (St. Louis, MO).

Batch reactor

The batch reactor consisted of a reaction vessel with steam jacket and stirring arm to provide uniform heating and mixing. The substrate was prepared by adding casein in 0.05M $NaH_2PO_4 \cdot H_2O$ buffer (pH 7) and adjusting to the desired pH with NaOH.

Formed-in-place (FIP) membrane

The membrane (DuPont Separation Systems, Seneca, SC, type F_4) was formed-in-place on 61 cm × 1.59 cm i.d. porous, stainless-steel tubes. The proprietary membrane-coating techniques and chemicals were provided by DuPont Separation Systems. The permeability of the membrane ranged from 1 to 1.5 (permeability = water flux/pressure). The optimum operation pH range was from 5 to 7 with temperature < 70°C.

Continuous FIP membrane reactor

Substrate for the feed vessel (6% casein) was prepared by suspending casein in 0.05M $NaH_2PO_4 \cdot H_2O$ and adjusting to pH 7 with NaOH. The reaction vessel (Fig. 1) was first filled to the required substrate volume from the feed vessel. After adjusting to 37°C, the enzyme solution was added. The pumping loop, consisting of a diaphragm positive displacement pump followed by a centrifugal pump, was started and adjusted to (predetermined) optimum pressure and flow rate. The pressure maximum was limited by the seal pressure of the centrifugal pump to about 3.4 atm. The mixture from the reaction vessel was pumped to the FIP membrane unit. The rejected stream (retentate) was then recirculated in the system. The permeate stream, consisting of peptides and amino acids small enough to pass through the membrane, was diverted through a flow meter to a suitable collection vessel. The level in the reaction vessel was maintained by adjusting the flow rate of fresh substrate from the feed tank to match the flow rate of permeate.

Nitrogen content

Nitrogen content was determined by micro-Kjeldahl analysis (Labconco 65000 rapid distillation unit, Labconco Corp., Kansas City, MO). All data were corrected for nonprotein nitrogen (NPN), which was determined as the initial nitrogen soluble in 10% trichloroacetic acid (TCA). The TCA-soluble nitrogen (N_{TCA}) in the product was measured as NPN. The % of TCA-soluble nitrogen or % conversion (X) was expressed as

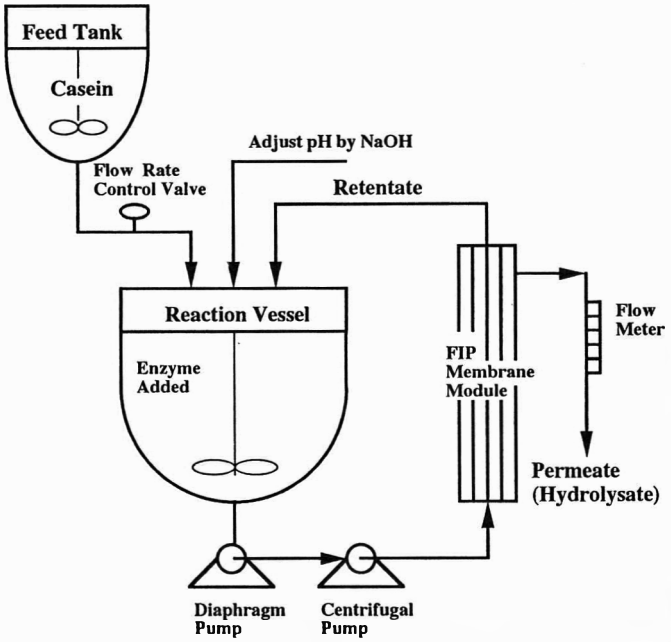

Fig. 1—Diagram of continuous FIP membrane reactor.

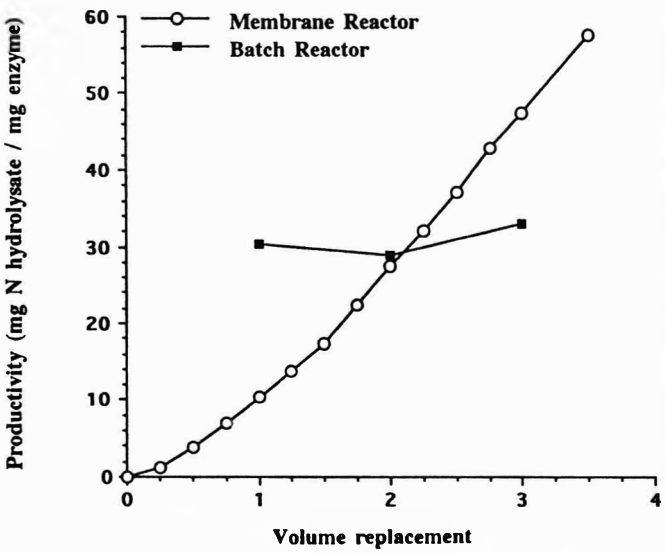

Fig. 2—Comparison of productivity of continuous FIP membrane reactor with that of batch reactor. Casein = 6% (w/v), E = 0.15 mg/mL, J = 18 mL/min and V = 5 L.

$$X = \frac{N_{TCA} - NPN}{TN - NPN} \times 100 \quad (1)$$

where TN = total nitrogen in the product.

Productivity study

For the batch reactor, productivity was defined as

$$P_{batch} = \frac{XS_c}{E} \quad (2)$$

where E = enzyme concentration (mg/mL); S_c = total nitrogen in unhydrolyzed substrate corrected for NPN.

For the continuous FIP membrane reactor, productivity was expressed as

$$P_i = \frac{\bar{P}Jt}{EV} \quad (3)$$

or

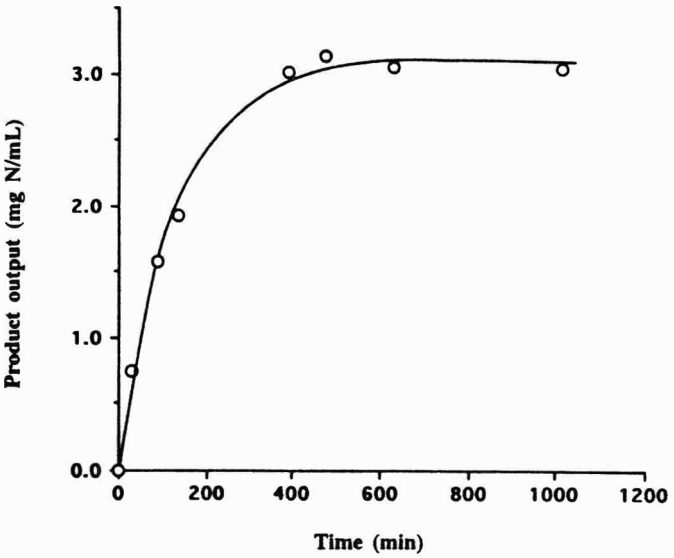

Fig. 3—Long-term stability of the continuous FIP membrane reactor.

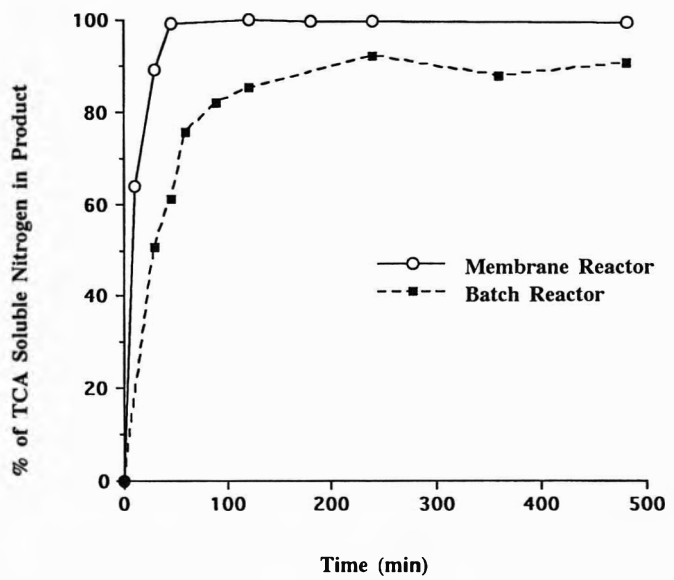

Fig. 4—Percentage of TCA-soluble nitrogen in products from the continuous FIP membrane reactor and batch reactor. Casein = 6% (w/v), E = 0.5 mg/mL, 37°C and pH 7.

$$P_c = \Sigma P_i \quad (4)$$

where P_i = instantaneous productivity; $\bar{P}$ = average product output (mg N/mL) in a time period t (min); J = flow rate (mL/min); P_c = cumulative productivity. P_c was calculated over all time periods studied. The unit of productivity was mass hydrolysate/mass enzyme (mg N/mg enzyme). A more complete description of productivity was given by Cheryan and Deeslie (1983).

Molecular weight distribution of the hydrolysates

Both high-performance size-exclusion chromatography (HPSEC) and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were used to analyze the molecular weight profile of hydrolysates (peptides and free amino acids) obtained from the FIP membrane unit. The HPSEC (Waters Associates, Milford, MA) was equipped with a Bio-Rad Bio-Sil SEC-125 column (600 × 7.5 mm), connected to a TSK precolumn (75 × 7.5 mm), operated at a column temperature of 25°C with a UV detector set at 280 nm. The mobile phase was 0.1M sodium phosphate (pH 6) with a flow rate of 1.0 mL/min.

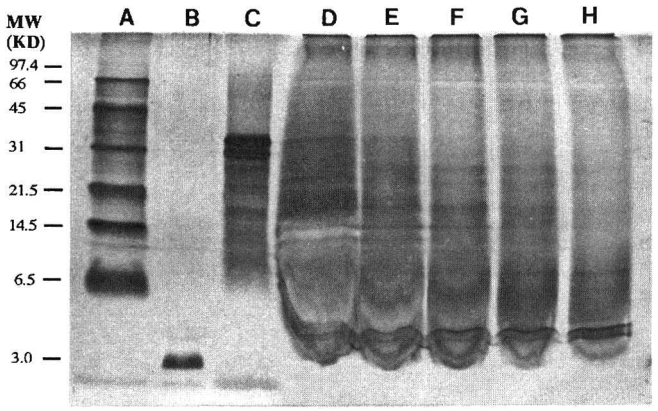

Fig. 5—SDS-PAGE using a 4% stacking gel and a 18% resolving gel. A and B = standard marker; C = casein; D, E, F, G, and H represent the hydrolysate withdrawn at 0.17, 1, 2, 4, and 8 hr from the batch reactor, respectively.

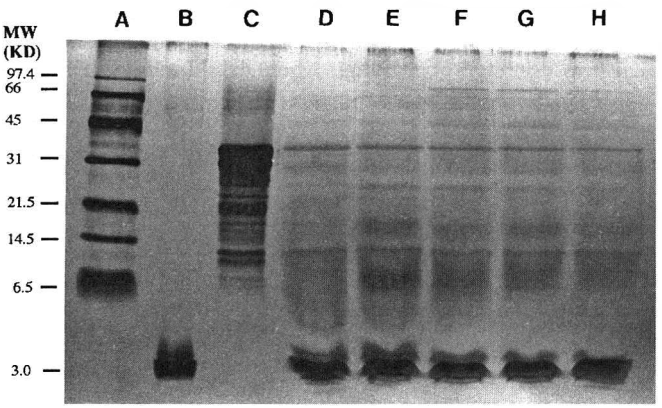

Fig. 6—SDS-PAGE using a 4% stacking gel and a 18% resolving gel. A and B = standard marker; C = casein; D, E, F, G, and H represent the hydrolysate (permeate) withdrawn at 0.17, 1, 2, 4, and 8 hr from the continuous FIP membrane reactor, respectively.

Functional and immunological properties

Solubility. Solubility of casein and hydrolysate was determined by a protein dispersibility index (PDI) method (AOCS, 1970) with slight modification. A 1% aqueous solution was blended for 10 min and adjusted to the desired pH with 2N NaOH or 2 N HCl. Then, the sample was centrifuged at 1500 × *g* for 10 min. The nitrogen content of the supernatant was determined. The percent solubility was calculated as:

$$\% \text{ solubility} = \frac{TN_s}{TN_i} \times 100 \qquad (5)$$

where TN_s is the total nitrogen in the supernatant and TN_i is the total nitrogen of initial 1% aqueous solution.

Moisture sorption. The method of Lang et al. (1981) using minidesiccators was used to determine the water adsorption isotherm of the hydrolysates and the intact casein.

Antigenicity. Immunologically active casein (IAC) was defined by reference to the unhydrolyzed casein using an inhibition enzyme-linked immunosorbent assay (ELISA) as described by Voller et al. (1980). Immunologically active whey protein (IAW) also could fit the definition by reference to unhydrolyzed whey protein. The ELISA procedure followed the method of Mahmoud et al. (1992). Casein antigen solutions at 10, 30, 100, 300, 1000, and 3000 ng/mL, prepared by using intact casein, were used to establish a standard curve by plotting logarithm of % inhibition of the ELISA signal (A/Ao) vs logarithm of casein concentration.

RESULTS & DISCUSSION

Continuous FIP membrane reactor

Productivity of the batch reactor was compared with that of the continuous FIP membrane reactor (Fig. 2) as a function of volume replacement. The productivity of the batch reactor was independent of volume replacement, whereas that of the continuous FIP membrane reactor had a linear increase as volume replacement increased. This was due to the fact that in the batch reactor each volume replacement required the same amount of enzyme (mg/mL). However, in the continuous FIP membrane reactor the enzyme was introduced only once in the beginning of the run. In this run higher productivity could be achieved for the continuous FIP membrane reactor after two or more volume replacements. This result also agreed with the findings by Mannheim and Cheryan (1990). They showed that continuous production of casein hydrolysate with ultrafiltration membrane systems allowed reuse of enzymes, thus resulting in higher productivity than with a conventional batch process.

Table 1—Antigenicity properties of hydrolysates from the continuous FIP membrane reactor

Sample	μg AC/g protein	μg IAW/g protein	% loss of IAC	% loss of IAW
Intact casein	1.03×10^6	1.67×10^5	—	—
H_2[a]	6.88×10^3	3.99×10^3	99.33	97.61
H_6[b]	8.10×10^3	4.17×10^3	99.21	97.50

[a] H_2: Hydrolysate of 2% casein + 0.5 mg/mL enzyme at 37°C and pH 7.
[b] H_6: Hydrolysate of 6% casein + 0.5 mg/mL enzyme at 37°C and pH 7.

Long-term stability of the FIP membrane reactor was evaluated by hydrolysis of 6% casein with 0.15 mg/mL alkali protease at pH 7 and 37°C (Fig. 3). The membrane reactor maintained steady TCA-soluble nitrogen production for at least 17 hr. Some enzyme leakage was indicated by detection of residue activity in the product (permeate) by SDS-PAGE (data not shown). However, studies of reactor stability and productivity (Fig. 2 and 3) did not show any significant effect of enzyme leakage. Furthermore, loss of reactor activity could be compensated by periodic adding of relatively small amounts of fresh enzyme into the reactor.

The percentage of TCA-soluble nitrogen in the products (hydrolysates) from the FIP membrane reactor were compared with those from the batch reactor (Fig. 4). A steady production of 99% TCA-soluble nitrogen was achieved after 45 min of operating the FIP membrane reactor, whereas only a maximum of 90% could be accomplished after 2 hr of batch reactor operation. Higher TCA-soluble nitrogen indicated the product from the continuous FIP membrane reactor contained a larger fraction of small peptides and amino acids. Based on SDS-PAGE analysis (Fig. 5 and 6) the product of the FIP membrane reactor had similar molecular weight (MW) profiles from 10 min to 8 hr, whereas the product of the batch reactor had different profiles at different hydrolysis times. This suggested that the molecular weight distribution in the hydrolysate was better controlled by the FIP membrane, thus creating hydrolysates with specific functional properties. The functional properties of a protein or its hydrolysate are governed by molecular size (Adler-Nissen, 1986). In batch reactors the difficulty of controlling the extent of reaction can result in nonhomogeneous products from batch to batch. (Cheryan, 1986).

Functional and immunological properties

Based on HPSEC analysis the hydrolysate contained primarily (45.7%) free amino acids and small peptides with MW < 500 daltons. Other fractions in the hydrolysate were: MW 500–1500, 22.0%; MW 1500–3000, 18.3%; MW 3000–4000, 12.3%; and MW greater than 4000, 1.7%. According to a clinical study by Seban et al. (1977), casein hydrolysate containing a polypeptide fraction with molecular weight >3,850, based on Sephadex G-25 filtration, resulted in antigenic responses. Poulsen and Hau (1987) concluded that peptides of <3,400 daltons, based on Biogel P-30 filtration, did not produce anaphylaxis. This product had 98% of the hydrolysate with molecular weight <4,000 which suggested that hydrolysis of casein by Protease Type XXIII in the membrane reactor should substantially reduce

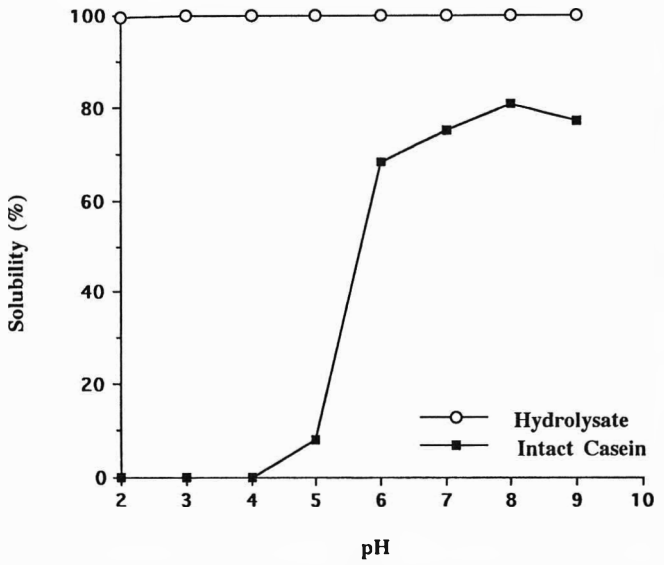

Fig. 7—Effect of pH on solubility of intact casein and its hydrolysate from the continuous FIP membrane reactor.

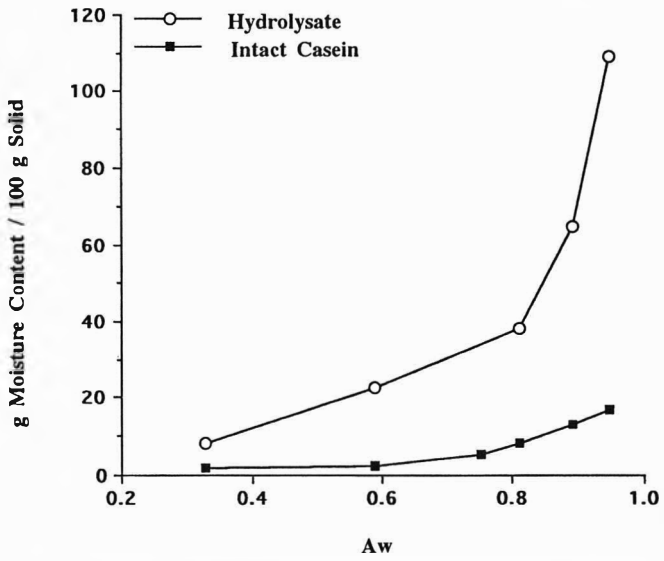

Fig. 8—Water sorption isotherms for intact casein and its hydrolysate from the continuous FIP membrane reactor at 21°C.

immunological reactivity. This was further supported by the antigenicity test (Table 1). The ELISA method was used to quantify casein or whey protein antigenic determinants (epitopes) remaining in the hydrolysate which retain the capacity to bind with casein (or whey protein)-specific antibodies. There were no significant differences in antigenicity properties by using substrate concentration of 2% or 6% casein. The results of ELISA indicated that the hydrolysates were >99% and 97% less antigenic on IAC and IAW than intact casein, respectively.

Based on SDS-PAGE analysis of the hydrolysates sampled at different hydrolysis times (Fig. 6), the heavy band areas ~3,000 daltons indicated that the major fractions of the hydrolysates were <4,000 daltons. Several bands located between 6,500 and 45,000 in SDS-PAGE analysis also reflected the small portions in HPSEC analysis. These bands suggested the presence of residual antigen in the permeate from the membrane reactor. Further reduction in antigenicity would require FIP membranes with lower permeability than the membrane we used.

The effect of pH on solubilities of intact casein and the hydrolysate for the FIP membrane reactor were compared (Fig. 7). Intact casein showed 70% to 85% solubility between pH 6 and 9 but very low solubility at pH < 5. The hydrolysate was completely soluble at all pH values indicating the potential for using this hydrolysate in a wide range of products, including acidic beverages.

Water sorption isotherms for intact casein and its hydrolysate at 21°C were compared (Fig. 8). Water sorption of the hydrolysate increased 4 to 6.5 times at water activity of 0.35–0.95 as compared to intact casein. This suggested the hydrolysate could be used as an additive in intermediate-moisture foods to bind water and control texture.

CONCLUSION

THE FIP MEMBRANE REACTOR was a stable and useful way to continuously produce a uniform casein hydrolysate with specific functional and immunological properties. Antigenicity reduction correlated with the molecular weight profile of the hydrolysate. In addition to being more efficient than batch operations, membrane reactors provide better control of properties of the hydrolysate by selection of size of peptide permeating the membrane. FIP membranes should be especially useful for this purpose, since a wide range of membrane permeabilities can be manufactured.

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Corn Syrup Oligosaccharide Effects on Sucrose Crystallization

P. TJURADI and R.W. HARTEL

ABSTRACT

The components of corn syrup (oligosaccharides with different degrees of polymerization) have long been hypothesized to have different effects on sucrose crystallization. Corn syrups were fractionated to produce four fractions, each containing a different range of oligosaccharides. Each fraction decreased the solubility concentration of sucrose to the same extent as the original corn syrup, except for the lowest DP fraction which caused a lower sucrose solubility in water. Each fraction also reduced the growth rate of sucrose crystals compared to the pure system. However, at equivalent molar levels, all corn syrup fractions had the same effects on reducing crystal growth.

Key Words: sucrose, crystallization, corn syrup, oligosaccharides, crystal growth

INTRODUCTION

CORN SYRUPS have found wide use in the food industry as a result of their unique properties and relatively low cost. For the food manufacturer, important uses of corn syrup are to control sucrose crystallization, increase viscosity and reduce sweetness. Corn syrup has been used in confections, baked products, preserves, and frozen desserts to control crystallization (McDonald, 1984), and enable development of appropriate properties in products.

In the confectionery industry, crystallization of sucrose is controlled by adding corn syrup. Thus, the proper crystal size distribution in products such as fondants, grained toffee, and caramels (McDonald, 1984) can be produced. This is crucial since crystal size distribution is important in determining overall texture and physical properties.

The presence of other sugars or other compounds in food products considerably affects sucrose crystallization, through reduction of solubility, inhibition of nucleation and growth rates and crystal morphology changes (Smythe, 1967, 1971; Burrill, 1972; Van Hook, 1981; Hartel and Shastry, 1991). Quantitative data have shown the inhibitory effects of glucose and invert sugar on sucrose crystal growth rates (Smythe, 1967). Dextrans in sugar cane decrease sucrose crystal growth rate and alter crystal habit (Sutherland and Paton, 1969; Mantovani et al., 1976; Kelly and Mak, 1977; Kelly et al., 1981). These effects have been related to preferential adsorption of dextrans on certain faces of the crystal, which slows growth rates of individual faces and thus, alters crystal shape (Mantovani et al., 1976; Morel du Boil, 1986).

The addition of corn syrup to sucrose in fondant manufacture resulted in decreased crystal growth rate and crystal size in the final product (Bamberger et al., 1980). They hypothesized that this effect was due to (1) the effect of long chain polymers on viscosity, which would reduce the rate of mass transfer, and (2) the adsorption of glucose units on the sucrose crystal lattice. They also suggested that lower DE corn syrups (DE 37 or 42) were more effective at reducing sucrose crystal growth than were higher DE corn syrups (62 DE), and that this was attributable to differences in content of longer-chain oligosaccharides. However, no detailed investigations on specific effects of oligosaccharides from corn syrup have been reported.

Our objectives were to determine the effects of oligosaccharides in corn syrups on solubility of sucrose in water, the rate and extent of sucrose crystallization, and final crystal morphology. Such information will help in understanding the effects of components of corn syrups in determining crystallization in sugar confectionery and other food products. Understanding these effects would help in development of new products as well as process operation and control.

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MATERIALS & METHODS

Corn syrup fractionation

Two types of corn syrup solids were used: 35 DE (Star-Dri® 35 R) and 42 DE (Star-Dri® 42 F) corn syrup solids, both supplied by A.E. Staley Manufacturing Company, Decatur, IL. Using a technique developed by the manufacturer, corn syrup from each source was fractionated by acetone into four fractions with different carbohydrate contents. Each fraction was named according to the average degree of polymerization (DP) of glucose molecules, determined based on the proximity of the average molecular weight of each fraction to the molecular weight of the nearest integer polymer. Fractions were thus classified by the original corn syrup (35 or 42 DE) and the average DP as: 35 $\overline{DP}$ 15, 42 $\overline{DP}$ 14, 35 $\overline{DP}$ 7, 42 $\overline{DP}$ 7, 35 $\overline{DP}$ 5, 42 $\overline{DP}$ 4, 35 $\overline{DP}$ 1, and 42 $\overline{DP}$ 1.

Corn syrup solids (both 35 and 42 DE) were fractionated using an acetone precipitation process. Corn syrup solids were dissolved in acetone and water, which caused preferential precipitation of certain oligosaccharides, depending on the acetone/water ratio. The method of fractionating corn syrup solids into four distinct fractions is illustrated diagrammatically (Fig. 1). Once fractionation was completed, freeze-dried samples of fractions were analyzed by high performance liquid chromatography (HPLC) to determine the range of oligosaccharides in each fraction and by gel permeation chromatography (GPC) for the average molecular weight.

Solubility of sucrose in the presence of corn syrup or its fractions

In order to prepare sucrose solutions with constant supersaturation, the solubility of sucrose in the presence of corn syrup fractions was determined. Into each of six 500-mL bottles, 100g deionized water were added. This was followed by addition of 0, 10, 20, 30, 40, and 50g of either corn syrup solids or its fractions and excess analytical reagent grade sucrose (Mallinkrodt, Paris, KY). All caps were screwed tightly before being placed in a rotating water bath maintained at 30 or 60°C. Solutions were rotated gently to provide constant stirring. The refractive index change of each solution was followed every day for 4 to 5 days, or until no further changes in refractive index were observed.

A Bausch & Lomb refractometer (Rochester, NY) with constant temperature water circulation (20°C) was used to measure refractive index. A sample solution was taken from the equilibrium system by pipetting. The clear, saturated sample solution was placed directly on the prisms of the refractometer and analyzed at 20°C. For each sample, the average of three readings was taken as the refractive index. We assumed that corn syrup solids have little effect on altering total solids readings from refractometers at the low addition levels studied. The concentration of sucrose was determined by subtracting measured total solids from the amount of corn syrup added. Solubility was expressed as g sucrose/100g water. Note that this measure of solubility concentration is not exact, due to differences in refractive index of sucrose and corn syrup solids, but gave a reasonable estimate of sucrose solubility.

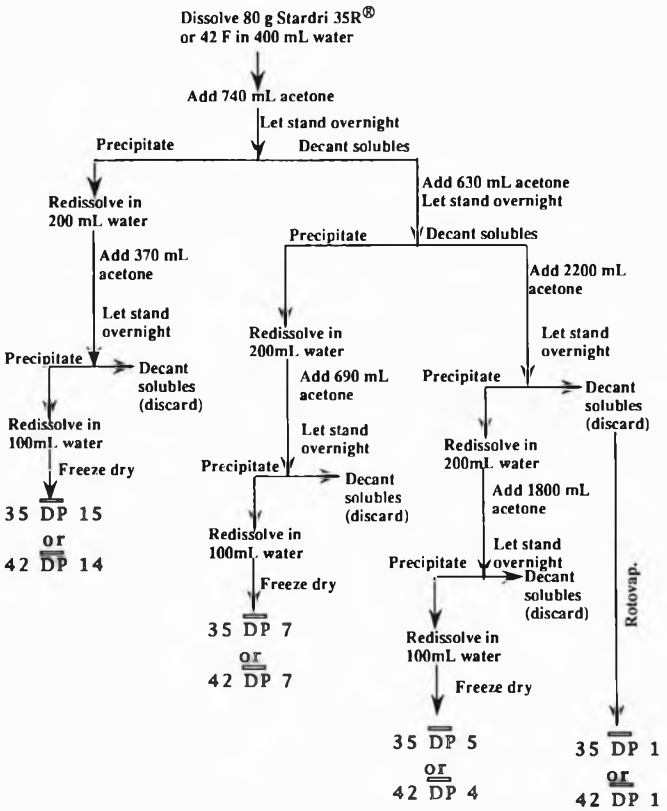

Fig. 1—Corn syrup fractionation technique.

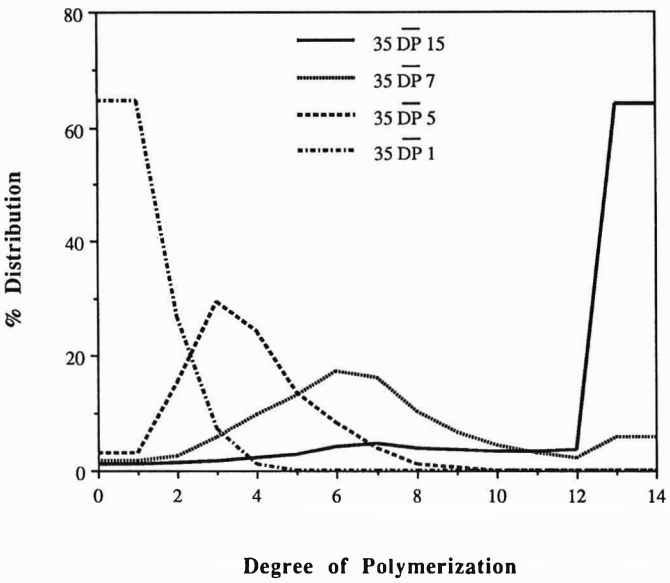

Fig. 2—Distribution of oligosaccharides (by degree of polymerization, DP) in 35 DE corn syrup fractions.

Crystal growth under stagnant conditions

Sucrose crystal growth in the presence of corn syrup solids or their fractions was studied at 30 or 60°C. All solutions were prepared at equivalent sucrose supersaturation ratio of 1.46 ± 0.02 based on concentration, g sucrose/100g water. Supersaturation ratio was defined as the ratio of actual sucrose concentration in solution to the solubility concentration (g sucrose/100g water) at that temperature. Initially, an undersaturated sucrose solution was prepared by dissolving analytical reagent grade sucrose crystals (Mallinckrodt, Paris, KY) in deionized water. Either corn syrup solids or one of the fractions was incorporated at 5, 10, or 20% (w/w) of the sucrose level and the solution concentrated to equivalent supersaturation in a rotary evaporator.

Seeds were prepared by precipitation of sucrose from solution through addition of methanol. Seeds used throughout the experiment were between 11.5 μm and 19.8 μm (mean size of 15.6 μm), as measured by video image analysis software (Java, Jandel Scientific, Sausalito, CA). A small amount of seeds was placed onto the sample stage and spread with a small drop of methanol in order to completely disperse the seeds. The methanol on the stage was dried for 20 to 30 min before use. The sample stage was then placed on a second, temperature-controlled stage with water bath set at either 30° or 60°C. The supersaturated solution was then poured into the sample stage and covered with a microscope slide. The two stages were placed on the platform of a Nikon microscope (Optiphot, Nippon Kogaku USA Inc., Garden City, NY), where the growth of sucrose crystals was followed by a video camera (Cohu, model 4815-2000/0000) connected to video image analysis software (Java, Jandel Scientific, Sausalito, CA). Projected area for each crystal was converted to equivalent circular diameter to generate a crystal size distribution, from which mean size and variance were determined. Mean growth rate was determined as the rate of change of mean size with time. Further details of the experimental technique may be found in Tjuradi (1994).

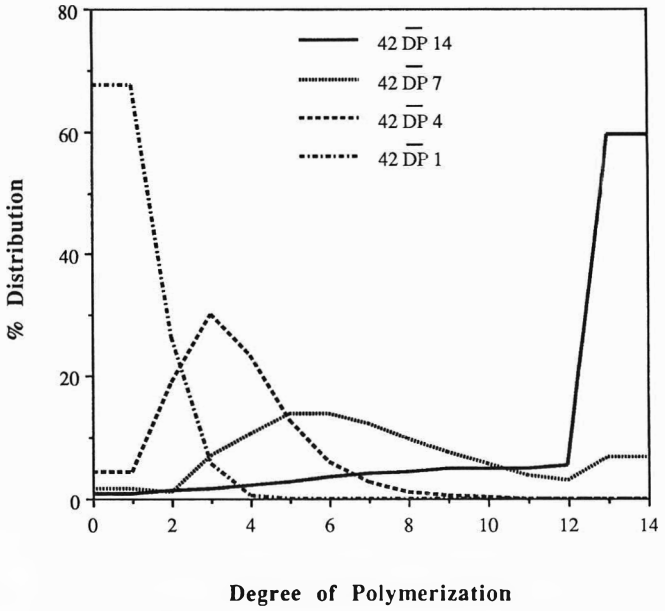

Fig. 3—Distribution of oligosaccharides (by degree of polymerization, DP) in 42 DE corn syrup fractions.

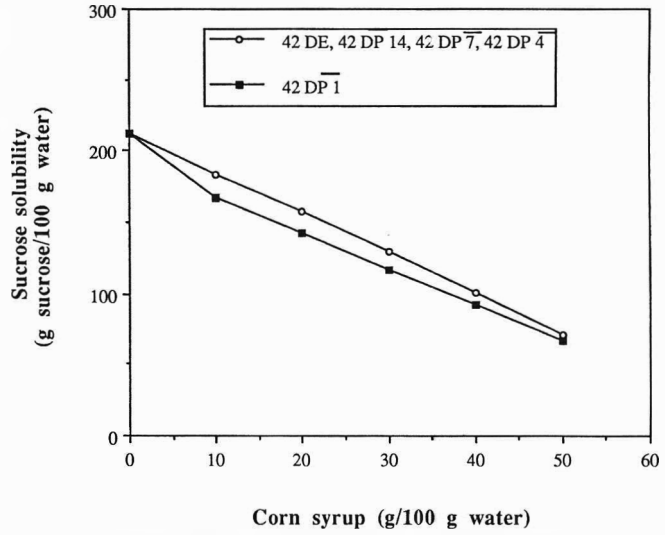

Fig. 4—Solubility of sucrose in the presence of 42 DE corn syrup solids and its fractions (at 30°C).

Modification of sucrose crystal shape in the presence of corn syrup

Changes of sucrose crystal morphology during growth in the presence of corn syrup solids and their fractions at 30° and 60°C were also observed. The images of crystals grown in the presence of corn syrup solids and their fractions at each condition were visually compared to those grown in pure sucrose solution.

Table 1—Average molecular weight of 35 and 42 DE corn syrup fractions

Sample	Mw (g/mole)
35 DP 15	1995
35 DP 7	998
35 DP 5	592
35 DP 1	225
42 DP 14	1738
42 DP 7	944
42 DP 4	547
42 DP 1	223

Table 2—Reduction in sucrose growth rate in the presence of 35 DE corn syrup solids and its fractions at each addition level as compared to pure sucrose growth (30°C, S=1.46)

Impurities added	Reduced growth rate by
5%, 35 DE	0.39
5%, 35 DP 15	0.52
5%, 35 DP 7	0.54
5%, 35 DP 5	0.58
5%, 35 DP 1	0.77
10%, 35 DE	0.41
10%, 35 DP 15	0.60
10%, 35 DP 7	0.67
10%, 35 DP 5	0.70
10%, 35 DP 1	0.82
20%, 35 DE	0.57
20%, 35 DP 15	0.64
20%, 35 DP 7	0.69
20%, 35 DP 5	0.72
20%, 35 DP 1	0.84

Table 3—Reduction in sucrose growth rate in the presence of 42 DE corn syrup solids and its fractions at each addition level as compared to pure sucrose growth (30°C, S=1.46)

5%, 42 DE	0.34
5%, 42 DP 14	0.40
5%, 42 DP 7	0.55
5%, 42 DP 4	0.62
5%, 42 DP 1	0.70
10%, 42 DE	0.41
10%, 42 DP 14	0.48
10%, 42 DP 7	0.61
10%, 42 DP 4	0.63
10%, 42 DP 1	0.72
20%, 42 DE	0.60
20%, 42 DP 14	0.64
20%, 42 DP 7	0.71
20%, 42 DP 4	0.73
20%, 42 DP 1	0.85

Table 4—Reduction in sucrose growth rate in the presence of 10% 35 DE corn syrup solids and its fractions as compared to pure sucrose growth (60°C, S=1.46)

Impurities added	Reduced growth rate by
10% 35 DE	0.56
10%, 35 DP 15	0.61
10%, 35 DP 7	0.67
10%, 35 DP 5	0.77
10%, 35 DP 1	0.85

RESULTS & DISCUSSION

Corn syrup fractionation

Fractionation of each Star-Dri 35 R and 42 F corn syrup using acetone resulted in eight corn syrup fractions. The amount of 35 $\overline{DP}$ 15 and 42 $\overline{DP}$ 14 powder collected in one course of fractionation was about 14g (17.5% from 80g of initial corn syrup solids). The amount of 35 $\overline{DP}$ 7 and 42 $\overline{DP}$ 7 insoluble powder collected in one course of fractionation was about 10g (12.5% from 80g of initial corn syrup solids). The amount of 35 $\overline{DP}$ 5 and 42 $\overline{DP}$ 4 powder collected in one course of fractionation was about 8g (10% of 80g initial corn syrup solids). The amount of 35 $\overline{DP}$ 1 and 42 $\overline{DP}$ 1 fractions collected in one course of fractionation (before removing water and acetone) was 60% of the initial 80 g corn syrup solids. Fractionated 35 DE (Fig. 2) and 42 DE (Fig. 3) corn syrup solids contained different oligosaccharide composition. The 35 $\overline{DP}$ 15 and 42 $\overline{DP}$ 14 fractions were composed primarily of glucose polymer chains of DP 12 and above. The $\overline{DP}$ 7 fractions were made up mostly of glucose polymers from DP 2 to 12. On the other hand, 35 $\overline{DP}$ 5 and 42 $\overline{DP}$ 4 fractions contained mostly glucose polymer chains from DP 1 to 8. $\overline{DP}$ 1 fractions consisted primarily of glucose and maltose (DP 1 and 2).

GPC analysis gave average molecular weights (Mw) of each corn syrup fraction (Table 1). Average molecular weights of each fraction from 35 and 42 DE corn syrups, with the exception of 35 $\overline{DP}$ 15 and 42 $\overline{DP}$ 14 fractions, were similar. The similarity of different corn syrup solid fractions was due to the chemical nature of the fractionation process, where separation was based on solubility of different molecular weight species in acetone-water.

Solubility of sucrose in the presence of corn syrup or its fractions

Solubility of sucrose was depressed in the presence of 42 DE corn syrup or any of its fractions (Fig. 4). This was most likely due to the competition between sucrose and corn syrup components for hydrogen bonding sites with water molecules (Mullin, 1971). Essentially identical results were found for solubility of sucrose in the presence of 35 DE corn syrup solids or its fractions.

The solubility of sucrose in the presence of either corn syrup solids or any of their fractions, except 35 $\overline{DP}$ 1 and 42 $\overline{DP}$ 1 fractions, was essentially equivalent. The influence of intact corn syrup solids and the oligosaccharide fractions on solubility of sucrose were similar for both 35 and 42 DE corn syrups. On the other hand, the presence of both 35 $\overline{DP}$ 1 and 42 $\overline{DP}$1 fractions in solution, depressed the solubility of sucrose even further compared to intact corn syrup solids or the other fractions. The reason for this was most likely that the number of glucose molecules per gram of 35 $\overline{DP}$ 1 and 42 $\overline{DP}$ 1 fractions added to sucrose solution was significantly higher than for the other fractions since addition levels were on a weight basis.

An increase in amount of corn syrup fractions added to the solution further decreased the solubility of sucrose, just as for intact corn syrup. The solubility of sucrose in the presence of 35 DE corn syrup solids and its fractions increased when the solution was held at 60°C, as compared to 30°C (data not shown). However, the same effect of depressed solubility with increasing fraction addition was observed.

Crystal growth under stagnant conditions

The rate of sucrose crystal growth in pure sucrose solution under static conditions was 0.68 μm with a variability of 0.05 μm/min. When corn syrup solids were introduced, the growth velocity of sucrose decreased significantly. Extent of reduction in growth rates were compared in the presence of 35 DE corn syrup solids and its fractions at 30°C (Table 2), 42 DE corn syrup solids and its fractions at 30°C (Table 3), and 35 DE corn syrup solids and its fractions at 60°C (Table 4). The tables can be used to determine growth rate at any condition by multiplying the growth reduction factor by the pure sucrose growth rate of 0.68 μm/min. Statistical analysis showed that the growth rate in the presence of corn syrup or its fractions was significantly ($p < 0.0001$) lower than for pure sucrose.

Potential mechanisms for sucrose growth inhibition by corn syrup fractions are (Hartel and Shastry, 1991): (1) Corn syrup molecules impede diffusional motion of sucrose molecules to the crystal surface and thereby, slow down crystal growth. (2) Corn syrup molecules adsorb to the crystal surface and inhibit incorporation of sucrose molecules into the crystal lattice. In such case, sucrose molecules would have to displace the oligosaccharide molecules in order for growth to occur. (3) Corn syrup molecules are incorporated into the sucrose crystal lattice.

There were significantly more molecules in the $\overline{DP}$ 1 fractions, since addition levels at equal weight were compared.

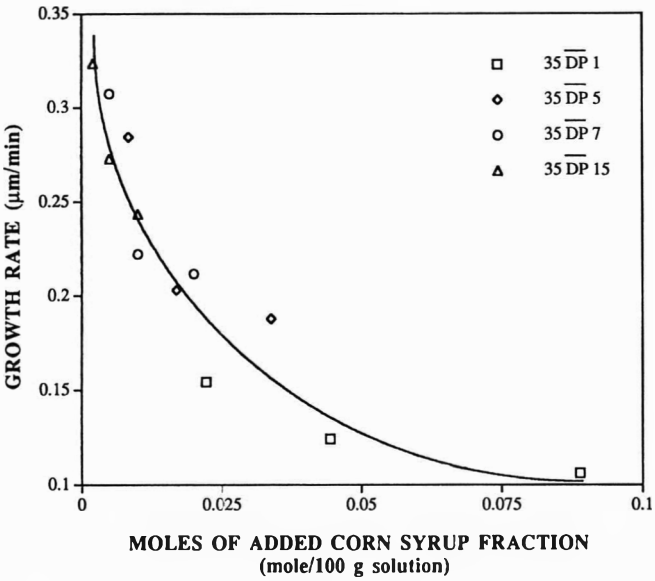

Fig. 5—Effects of molar addition of 35 DE corn syrup solids and it fractions on mean sucrose crystal growth rate.

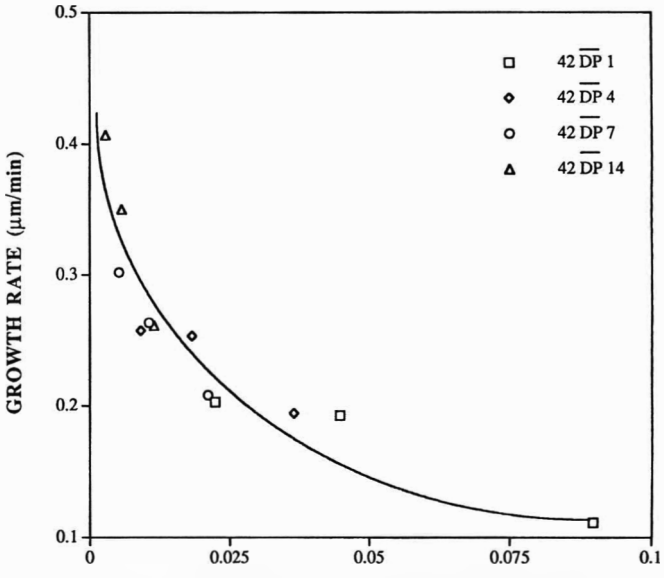

Fig. 6—Effects of molar addition of 42 DE corn syrup solids and it fractions on mean sucrose crystal growth rate.

Based on the average molecular weight for each fraction (Table 1), an estimate of the number of moles present for each addition level was calculated. Growth rates were compared based on this estimated number of moles across each fraction and each addition level (Fig. 5 for 35 DE and Fig. 6 for 42 DE corn syrup solids). These results strongly suggest that the important parameter affecting sucrose crystal growth was the number of moles added and not the average DP of each fraction. Although experimental deviations exist, the general shape of each curve showing inhibition of sucrose crystallization for each fraction was the same. In at least one specific case, three different fractions added at the same molar level had exactly the same effect on sucrose crystal growth rate. For example (Fig. 6), addition of 0.01 moles of DP 4 (5% weight addition), DP 7 (10% weight addition) and DP 14 (20% weight addition) gave almost the same mean sucrose growth rates, within experimental error.

Based on these results, addition of higher DE corn syrups with a larger number of smaller molecules would be expected to inhibit sucrose crystallization more than a corresponding amount (by weight) of a lower DE corn syrup. This result did not confirm the results of Bamberger et al. (1980), who found that lower DE corn syrups (37 and 42 DE) were more effective crystallization inhibitors than higher DE corn syrups (62 DE). However, in that work, supersaturation of sucrose was not maintained constant, which may explain the difference in results since growth rate also depends on supersaturation.

Our results suggest the following hypothesized mechanism of crystal growth inhibition for corn syrup. The active glucose end of each oligosaccharide molecule adsorbs at the crystal surface, hindering incorporation of sucrose molecules into the crystal lattice. In order for a crystal to grow, sucrose molecules must displace the glucose end-molecules, due to the higher adsorption energy for sucrose. Apparently, longer chained oligosaccharides did not present additional hindrance to growth compared to glucose molecules. Experiments utilizing individual oligosaccharides are needed to verify this mechanism.

Modification of sucrose crystal shape in the presence of corn syrup

The addition of corn syrup solids or their fractions at different levels and temperatures influenced sucrose crystal morphology only slightly. The inclusion of 5% or 10% corn syrup solids (DE 35 and 42) and their fractions into sucrose solution at 30°C were not sufficient to alter sucrose crystal shape. However, at 20% addition of corn syrup solids and their fractions (except for $\overline{DP}$ 1) addition, small numbers of tiny needle crystals were formed. The inclusion of $\overline{DP}$ 1 fractions at any addition level, 5%, 10%, or 20%, did not appear to result in needle crystal formation. The shape of crystals grown in the presence of $\overline{DP}$ 1 fractions was similar to that of crystals grown in pure sucrose solution. The number of needle type crystals was significantly increased, when 10% of 35 DE corn syrup solids and its fractions were added to sucrose at 60°C. This may be due to dissimilarity of kinetic growth mechanisms of sucrose at 30°C and 60°C, since surface integration occurs more rapidly at 60°C (Van Hook, 1981). In addition, there was an increase in overall extent of crystal growth at 60°C, hence, shape changes were easier to see.

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Ms received 5/4/95; revised 7/20/95; accepted 7/24/95.

This work was performed through support of the National Confectioner's Association and the American Association of Confectionery Technologists. The assistance of Dr. Gary Day of A.E. Staley Manufacturing Co. and Scott Helstad of Cargill, Inc. are gratefully acknowledged.

Nonspecific Enzyme-Linked Immunosorbent Assay for Molds in Foods

R.K. YONG and M.A. COUSIN

ABSTRACT

A nonspecific enzyme-linked immunosorbent assay (ELISA) was developed to detect molds in foods by producing an antibody to a mixture of six common molds, *Aspergillus versicolor, Cladosporium herbarum, Fusarium poae, Geotrichum candidum, Mucor circinelloides*, and *Penicillium chrysogenum*. This antibody recognized these mold genera plus 10 others but not yeasts. Mold antigens added into Cheddar and cottage cheeses, fruit juices, nonfat dry milk, raisins, and yogurt could be detected. Molds (10^2 spores/g) inoculated into cottage cheese and yogurt and allowed to grow at 7 or 22°C were detected at 10^3 CFU/g. A nonspecific ELISA could be developed to detect general mold contamination of foods.

Key Words: molds, enzyme-linked, antibodies, immunosorbent, ELISA

INTRODUCTION

RESEARCH has been reported on estimating mold presence in foods by immunological methods, particularly enzyme-linked immunosorbent assays (ELISA) and their extensions, latex agglutination assays (Cousin et al., 1990; De Ruiter et al., 1993; Kamphuis and Notermans, 1992; Kamphuis et al., 1989a). Extracellular antigens to *Penicillium verucosum* var. *cyclopium*, *Mucor racemosus* and *Fusarium oxysporum* were used to develop an ELISA to detect those molds in fruits (Notermans and Heuvelman, 1985; Notermans and Soentoro, 1986a). The antibodies were not genus specific because *Penicillium* antibodies cross-reacted with antigens from species of *Aspergillus* and *Mucor* in addition to *Penicillium* (Notermans and Soentoro, 1986a). Likewise, *Fusarium* antibodies cross-reacted with antigens from species of *Aspergillus*, *Penicillium* and *Trichothecium* in addition to those of *Fusarium*. Lin and Cousin (1987) noted that antibodies to *Alternaria alternata* cross-reacted with antigens from species of *Alternaria*, *Colletotrichum*, *Epicoccum*, *Leptosphaerulina*, *Schizophyllum*, and *Trichoderma*. Antibodies to *Geotrichum candidum* only recognized antigens of other *Geotrichum* strains and antibodies to *Rhizopus stolonifer* only recognized antigens from *Rhizopus* and *Mucor* species. Further research showed that many of the antibodies produced in rabbits were generally genus specific or only reacted with antibodies of closely related genera (Cousin et al., 1990; Notermans et al., 1986b; Tsai and Cousin, 1990).

Mold antigens are glycoproteins associated with the cell wall or with extracellular polysaccharides produced during growth (De Ruiter et al., 1993; Notermans et al., 1986b; Tsai and Cousin, 1993). The molds' antigenic determinants are usually in the carbohydrate part of the glycoprotein; although, the protein portion can be important for some antibodies (Tsai and Cousin, 1993). β-D-Galactofuranoside is immunodominant in the *Aspergillus/Penicillium* antigens (Kamphuis et al., 1989b, Notermans et al., 1988b; Van Bruggen-van der Lugt et al., 1992). *Mucor/Rhizopus* antigens had 2-O-methyl-D-mannose residues at the immunodominant site (De Ruiter et al., 1994). Most mold antigens contained galactose, glucose and mannose; however, the immunodominant linkages of these sugars were not the same in each genus (Tsai and Cousin, 1993). Identification of molds to the genus level is possible; however, little has been reported on detection of general mold contamination of foods by ELISA.

Commercial latex agglutination tests using antibodies raised against *Aspergillus* and *Penicillium* species have been available in Europe (Braendlin and Cox, 1992; De Ruiter et al., 1993; Karman and Samson, 1992; Kamphuis and Notermans, 1992; Stynen et al., 1992; Van der Horst et al., 1992). One kit (Mould Reveal Kit, Eco-Bio, Genk, Belgium) with monoclonal antibodies produced to *Aspergillus fumigatus* could detect 13 other genera, but not some of the common genera from food, such as *Alternaria*, *Fusarium*, *Geotrichum*, *Mucor* and *Rhizopus* (Stynen et al., 1992). Robertson et al. (1986) produced an antibody to a mixture of five molds (*Alternaria alternata*, *Botrytis cinerea*, *Fusarium solani*, *Mucor piriformis* and *Rhizopus stolonoifer*) that are commonly found in tomatoes. This antibody recognized these five molds plus *Geotrichum candidum* and *Penicillium chrysogenum*. Those results showed that one antibody could recognize several mold antigens that had not been used to produce the antibody.

It would be useful to the food industry if a general ELISA could be developed to indicate the presence of molds in foods. Our objective was (1) to produce a nonspecific antibody to common mold genera that contaminate foods, namely, *Aspergillus versicolor*, *Cladosporium herbarum*, *Fusarium poae*, *Geotrichum candidum*, *Penicillium chrysogenum*, and *Mucor circinelloides*, and (2) to evaluate the antibody in an ELISA for its nonspecificity and sensitivity to various molds in foods.

MATERIALS & METHODS

Fungal cultures

Aspergillus versicolor ATCC 44605, *Cladosporium herbarum* ATCC 28987, *Fusarium poae*, *Geotrichum candidum* NRRL Y-552, (NRRL, United States Department of Agriculture, Peoria, IL), *Mucor circinelloides* NRRL 3614 and *Penicillium chrysogenum* ATCC 10106 (American Type Culture Collection, Rockville, MO) were used to immunize New Zealand white rabbits. Other species of molds and yeasts were used to determine the nonspecificity of the mixed antibodies: *Alternaria* species, *A. nidulans var. latus*, *A. fumigatus*, *A. clavatus*, *A. candidus*, *A. ochraceus*, *A. niger*, *A. terreus*, *A. petraki*, *P. urticae*, *P. italicum*, *P. expansum*, *P. frequentans*, *P. cyclopium*, *P. crustosum*, *P. lanosum*, *P. corylophilum*, *Cladosporium* species, *R. oligosporus· F. sporotrichoiodes*, *F. graminearum*, and *F. nivale*, were obtained from Dr. L.B. Bullerman, Dept. of Food Science & Technology, Univ. of Nebraska-Lincoln; *A. alternata*, *A. cucumerina*, *A. solani* #11888, *F. acuminatum* #116560, *F. culmorum* #11686, *F. moniliforme* #12456, *F. moniliforme* #12460, *F. moniliforme* NRRL 13564, *F. oxysporum* #11904, *F. oxysporum* #12569, *F. roseum 'Gibbosum'* #1564, *F. solani* #12561, *F. heterosporum* #11336, *Monascus* species #11417, *Mucor* species, *Pythium aphanidermatum*, *Schizophyllum commune*, *Leptosphaerulina briosiana*, *Epicoccum rinomas*, *Trichoderma*, *Trichoderma* species #11219, *Trichoderma viride* GA-36, and *Sordaria fimicola*, were obtained from Dept. of Botany & Plant Pathology, Purdue Univ., *Hansenula anomola* 67–455, *H. canadens* 1-1252, *Candida lipolytica*, *C. krusei*, *Saccharomyces cerevisiae* (Chablis), and *S. cerevisiae* (Champagne) were obtained from Dr. L.R. Beuchat, Dept. of Food Science, Univ. of Georgia; *F. avenaceum* R-8983, *F. culmorum* R-8545, *F. napiforme* M-5909, *F. nygamai* M-6126, *F. proliferatum* M-3446, *F. sambucinum* R-5463, *F. semitectum* R-3962, *F. tricinctum* T-429 were obtained from Dr. P.E. Nelson, Dept. of Plant Pathology, College of Agriculture, Fusarium Research Center, Pennsylvania State Univ.; *Aureobasidium pullulans*

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ATCC 16622, *A. flavus* ATCC 22546, *A. parasiticus* NRRL 26691, *A. repens* ATCC 48521, *Epicoccum nigrum* ATCC 48208, *Eurotium repens* ATCC 42689, were obtained from the American Type Culture Collection, Rockville, MO; *A. fischeri* NRRL 181, *A. wentii* NRRL 377, *A. aluteus* NRRL 402, *A. oryzae* NRRL 447, *A. parasiticus* NRRL 502, *A. parasiticus* NRRL 3145, *A. parasiticus* NRRL 13539, *A. soyae* NRRL 5594, *A. flavus* NRRL 13135, *A. foetidus*, *A. nomius* NRRL 13137, *A. tamarii* NRRL 20818, *Chaetomium globosom* NRRL 1669, *Phoma exigua* #120763, *Monascus ruber* #11596, *Trichothecium roseum* #12307, *Verticillium albo-atrum* NRRL 1204 were obtained from S. W. Peterson, United States Department of Agriculture, Microbial Properties Research, Peoria, Illinois; *Neosartorya fischeri* #1110483, *P. martensii* NRRL 1843, *P. oxalicum* NRRL 5202, *P. implicatum* WDC 15, *Talaromyces flavus* NRRL 13535, *Byssochlamys fulva* 5-6 were obtained from Dr. A.D. King Jr., United States Dept. of Agriculture, Albany, CA; *Botrytis aclada* #17465 and *B. cinera* #17432 were obtained from Dr. P.V. Nielsen, Institute of Biotechnology (IBT), The Technical Univ. of Denmark, Lyngby, Denmark; and *P. citrinum* 46-006-05, *P. aurantiogriseum* 02-057-06, *P. miczyskii* 46-141-13, *P. spinulosum* 46-104-08, *P. variabile* 02-072-16, *P. viridicatum* 02-057-09 were obtained from Dr. H. Gourama, Pennsylvania State Univ.-Berks Campus, Reading, PA.

Preparation of mold mycelium

For the preparation of mold mycelium, all strains were grown on potato dextrose agar slants (Difco Laboratories, Detroit, MI) at 25°C for 4 to 7 days. Spores were washed from the surface with 5 mL of sterile deionized water with 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO). Flasks containing 250 mL of brain heart infusion broth (Difco Laboratories) were inoculated with 0.1 mL of the spore suspensions and rotated at 140 rpm on a Lab-Line orbit environ-shaker (Lab-Line Instruments, Inc., Melrose Park, IL) at room temperature (≈23°C) for 3 to 14 days depending upon the mold used. Mold mycelia were autoclaved (Amsco American Sterilizer Co., Erie, PA) at 121°C for 10 min and collected by filtration through four layers of sterile cheese cloth. After four washes with sterile deionized water, the fungal mycelia were freeze-dried (Labcono Co., Kansas City, MO) and ground in a Wig-L-Bug (Vivadent Silamat Co., Amherst, NY) for 30 sec.

Preparation of mold spores

For the preparation of mold spores, strains of *A. versicolor*, *C. herbarum*, *F. poae*, *G. candidum*, *M. circinelloides*, and *P. chrysogenum* were grown on potato dextrose agar slants (Difco Laboratories). Spore suspensions (3 mL), washed from the slants, were spread uniformly over the surface of 150 mL of minimal agar (formula obtained from laboratory of Dr. John Hamer, Dept. of Biology, Purdue Univ.) containing per liter: 15 mL of nitrate salt solution containing 120g $NaNO_3$, 10.4g KCl, 10.4g $MgSO_4 \cdot 7H_2O$, 30.4g KH_2PO_4; 10g dextrose; 1 mL of 1000× trace elements containing 80 mL of double distilled H_2O, 2.2g $ZnSO_4 \cdot 7H_2O$, 1.1g H_3BO_3, 0.5g $MnCl_2 \cdot 4H_2O$, 0.17g $CoCl_2 \cdot 6H_2O$, 0.17g $CuSO_4 \cdot 5H_2O$, 0.15g $Na_2MoO_4 \cdot 2H_2O$, 0.5g $FeSO_4 \cdot 7H_2O$, 5.0g EDTA (Na_4); 1 mL of 1% thiamine; 50 µL of 0.05% biotin solution; 18g of agar; and water to 1L. The pH was adjusted to 6.5 with 1.0N NaOH. Minimal agar was autoclaved in roux culture bottles, spore suspensions were added, and incubation was at 25°C for 7 to 14 days. Spores were released from mycelia by washing with sterile deionized water containing 0.05% Tween 20 (Sigma Chemical Co.). The spore suspension was centrifuged (Beckman Instruments, Inc., Palo Alto, CA) at 8000 × *g* for 15 min. After washing the suspension 4 times with sterile deionized water followed by centrifugation, spores were suspended in sterile deionized water, placed in tubes, capped and refrigerated at 4°C.

Production of nonspecific antibody

New Zealand white rabbits were injected intramuscularly with 3 mL of buffered suspension containing a mixture of 6 molds: *A. versicolor* (17 mg), *C. herbarum* (17 mg), *F. poae* (17 mg), *G. candidum* (1.7 mg), *M. circinelloides* (1.7 mg), and *P. chrysogenum* (17 mg) in PBS that was emulsified in 1.5 mL Freund's complete adjuvant (Sigma Chemical Co.). Likewise, booster injections with a similar concentration of mold in 1.5 mL PBS that was emulsified in 1.5 mL Freund's incomplete adjuvant (Sigma Chemical Co.) were given weekly until the titer stabilized as determined by the indirect ELISA developed by Lin et al. (1986) as modified by Tsai and Cousin (1990). When the absorbance for the titer reached 10^5 or greater, rabbits were bled by heart puncture. The blood was left at room temperature for 1 hr to coagulate, and the clot was removed by centrifuging (Beckman) at 9000 × *g* for 10 min.

Purification of antibody

Rabbit antiserum was first centrifuged (Beckman) at 14000 × *g* for 15 min and membrane filtered (0.45 µm pore size from Nalgene, Rochester, NY) before loading onto the column. Five mL of Protein-A Sepharose 4B fast flow (Sigma Chemical Co.) were packed onto a C-10, 1 × 10 cm, bed volume 5 mL (Pharmacia Biotech Inc., Piscataway, NJ) column and equilibrated with 10 column volumes of the binding buffer (0.1M Na_2HPO_4 + 0.01% thimerosal; pH 7). Six mL of centrifuged and filtered antiserum were loaded onto the top of the Protein-A column. The flow rate was set at 50 mL/hr. The eluent was monitored by UV (Perkin-Elmer, Norwalk, CT) at 280 nm, and the unbound portion of 5 mL fractions/tube was collected with a fraction collector (Bio-Rad, Melville, NY). The antibody was eluted with a buffer containing 0.1M citrate buffer (pH 3.0) + 0.01% thimerosal (Sigma Chemical Co.) and 2.5 mL fractions/tube were collected. Immediately after each antibody tube was collected, 2.5 mL of 0.1M Na_2HPO_4 (pH 9.2) + 0.01% thimerosal were added to each tube to neutralize pH. Protein-A fractions were checked for antibodies by using the indirect ELISA (Lin et al., 1986; Tsai and Cousin, 1990). Antibody fractions were pooled and dialyzed overnight (Baxter Diagnostics Inc., McGaw Park, IL, MW cutoff:14,000 Da) against distilled water at 5°C using 3 changes of water. The dialyzed antibody was then freeze dried and stored at −20°C.

Antibody conjugated with horseradish peroxidase

The antibody was coupled to peroxidase using the periodate method of Tijssen and Kurstak (1984) with the following modifications. For activation of peroxidase, 5 mg of peroxidase (EC 1.11.1.7, Horseradish, Type VI, Sigma Chemical Co.) were added to 0.5 mL of freshly prepared 0.1M $NaHCO_3$ (pH 8.1) followed immediately by addition of 0.5 mL of 12 mM $NaIO_4$. This was incubated in total darkness at room temperature for 1.5 hr. For the conjugation step, 15 mg of antibody dissolved in 1.5 mL of 0.1M Na_2CO_3 (pH 9.2) were added to the activated peroxidase followed by 0.42g of dry Sephadex G-25 (Sigma Chemical Co.; bead size 50–150µ) with incubation at room temperature for 3 hr. The conjugate was eluted from the Sephadex G-25 with 7.5 mL of 0.1 M Na_2CO_3 (pH 9.2) making the final volume 10 mL. For stabilization, 0.5 mL of 1/20 volumes of freshly prepared sodium borohydride (Sigma Chemical Co., 5 mg/mL) in 0.1 mM NaOH was added and incubated at room temperature for 30 min. Next a 3/20 volume of sodium borohydride was added and incubated for 30 min at room temperature. Lastly, to precipitate the conjugate, an equal volume of saturated ammonium sulfate, which was adjusted to pH 7.2 with 6N ammonium hydroxide, was added and then incubated at 5°C for 2 hr. This precipitated conjugate was centrifuged (Beckman Instruments) at 9000 × *g* for 20 min and pellets were collected and redissolved in 2 mL phosphate buffer saline (PBS). This was dialyzed at 5°C overnight (Baxter Diagnostics) using 1 L PBS that was changed three times. The conjugate was then applied to a C-10 column, (1 × 10 cm, bed volume 5 mL, Pharmacia Biotech) packed with ConA-Sepharose (Sigma Chemical Co.) with PBS used as both the equilibrating and starting buffer. This was pumped by a peristaltic pump (Rainin Instrument Co. Inc., Woburn, MA) to remove free antibody. Conjugate was desorbed using 0.05M methyl-α-D-mannopyranoside (Sigma Chemical Co.) in PBS. Bound antibody fractions were collected, concentrated by the Micro-ProDiCon ultrafiltration system (Spectrum Medical Industries, Houston, TX) and freeze-dried for later use.

Food samples with added mold

Food samples including yogurt, Cheddar cheese, cottage cheese, nonfat dry milk, apple juice, grape juice, and raisins were obtained from local retail stores. Freeze-dried molds were added individually to these food products at concentrations from 0 to 1 mg/mL. Food products were diluted from 7- to 10-fold with 50 mM citrate buffer (pH 5.0). All samples were homogenized with a Stomacher (Seward Medical, Cincinnati, OH) for 30 sec.

Food samples inoculated with molds

From each individual spore suspension 0.2 mL (≈ 1 × 10^3 spores/mL) of the mold mixture (*A. versicolor*, *C. herbarum*, *F. poae*, and *P. chrysogenum*) was inoculated into 20g of each dairy product and incubated at 7 and 22°C for up to 22 days. Samples were analyzed in duplicate every 2 to 4 days by weighing and transferring 20g of each food into 120 mL of 50 mM citrate buffer (pH 5.0) and homogenizing them with the Stomacher (Seward Medical) for 30 sec. Samples were then analyzed by the double sandwich ELISA (Lin et al., 1986; Tsai and Cousin, 1990).

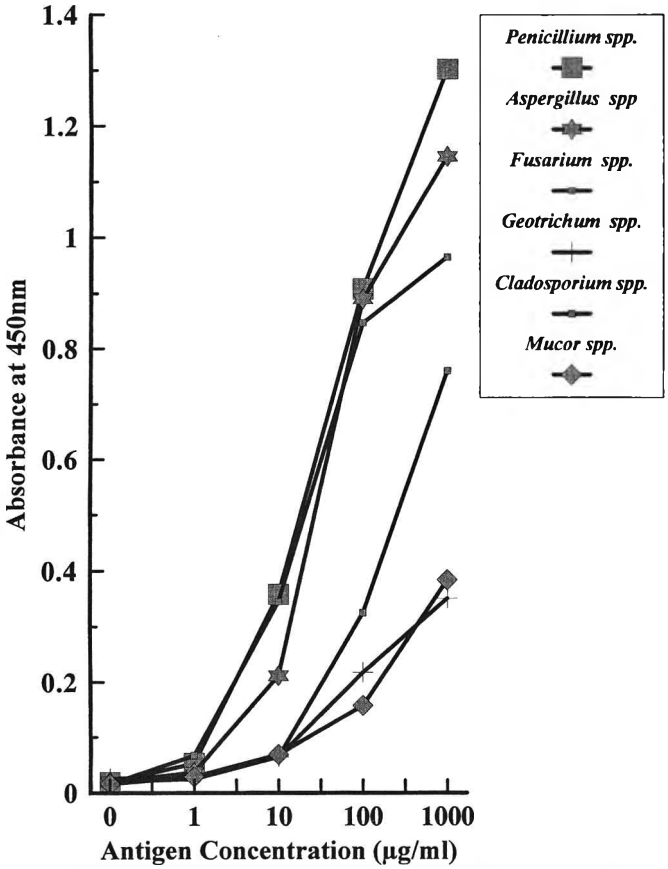

Fig. 1—ELISA for six molds (*A. versicolor, C. herbarum, F. poae, G. candidum, M. circinelloides,* and *P. chrysogenum*) in citrate buffer.

Dilution plating

All samples were analyzed by surface plating appropriate dilutions on dichloran rose bengal agar (Oxoid, Ogdensburg, NY) containing 100 µg/mL of chloramphenicol in 100 × 15 mm sterile petri plates (Mislivec et. al., 1992). Plates were incubated upright at 22°C for 5 days. Molds were counted and reported as colony forming units per gram (CFU/g) of food sample.

ELISA methodology for antigen detection

Double-sandwich ELISA. The double-sandwich ELISA of Lin et al. (1986) as modified by Tsai and Cousin (1990) was used to detect antigens in all samples and to test antigen-antibody cross-reactivity. Briefly, 200 µL of antibody diluted in carbonate coating buffer pH 9.6 (1.59 g sodium carbonate and 2.93 g sodium bicarbonate/L deionized water) were added to each well of a microtiter plate (Dynatech Laboratories, Inc., Alexandria, VA) and incubated at 37°C for 2 hr. Each plate was washed 4 times by an ELISA washer (Model EL-401, Bio-Tek Instruments, Inc., Burlington, VT) with PBS-Tween. Then 200 µL of sample in buffer were added to the wells, and plates were incubated at 37°C for 4 hr. Then ELISA plates were washed four times with PBS-Tween, and 200 µL of the antibody-peroxidase conjugate were added to each well. Plates were incubated overnight at 5 °C, and then washed 4 times with PBS-Tween. One hundred µL of 5-aminosalicyclic acid (Sigma Chemical Co.) [0.8 mg/mL, pH 6.0 with 0.05% H_2O_2 (Fischer Scientific, Fair Lawn, NJ)] were added to each well to form color and incubated at room temperature for 30 min. To stop the reaction, 50 µL of 1N NaOH were added to each well, and the absorbance was measured at 450 nm with an ELISA reader (Model EL-307, Bio-Tek Instruments, Inc., Burlington, VT).

Indirect ELISA. An indirect ELISA was used to measure the titer in the rabbit serum. Two hundred µL of carbonate coating buffer containing 0.1 mg/mL of mold mycelium were added to each well, and plates were incubated at 5°C overnight. The plates were washed four times with PBS-Tween, and 200 µL of serum [diluted in PBS-Tween plus 1% Bovine Serum Albumin, (BSA); Sigma Chemical Co.] were added to each well. The plates were incubated at 37°C for 2 hr. After washing each plate four times, 200 µL of goat anti-rabbit IgG peroxidase conjugate (Boehringer Mannheim Biochemicals, Indianapolis, IN) diluted 1:5000 in PBS-Tween plus 1% BSA were added followed by incubation at room temperature for 2 hr. The rest of the procedure followed the double-sandwich ELISA described above. ELISA readings were plotted against the logarithmic dilution of serum. The serum titer was calculated as the reciprocal of the dilution of serum that gave an ELISA reading three times higher than that of the control.

RESULTS & DISCUSSION

Sensitivity of general ELISA

The detection range for the mixed antibody was 1 µg/mL to 0.1 mg/mL depending on the type of mold (Fig. 1). The lowest detectable levels or sensitivity of assay for each mold for the mixed antibody were: 1 µg *A. versicolor*/mL, 10 µg *C. herbarum*/mL, 0.1 µg *F. poae*/mL, 10 µg *G. candidum*/mL, 10 µg *M. circinelloides*/mL, and 0.1 µg *P. chrysogenum*/mL. Tsai and Cousin (1990) reported a sensitivity range of 1 ng to 1 µg/mL for the five individual molds that included all except *F. poae*. Sensitivities were 1 µg/mL for *M. circinelloides*, *A. versicolor*, *P. chrysogenum*, and *C. herbarum*, and < 1 ng/mL for *G. candidum* (Tsai and Cousin, 1990). Sensitivity of *F. poae* was 0.1 µg/mL.

Of the six molds injected into the rabbit to produce the common antibody, three genera, *Aspergillus*, *Penicillium*, and *Fusarium*, showed high activity, with *P. chrysogenum* having the highest sensitivity or lowest detection level followed by *A. versicolor*, and *F. poae* (Fig 1). *Cladosporium* showed a lower activity compared to these three genera but had a higher sensitivity when compared to the *Geotrichum* and *Mucor* species. *G. candidum* and *M. circinelloides* were injected into rabbits at 1/10 the concentration of the other genera because a previous antibody produced with an equal level of all genera gave a reaction only with antigens from these two genera. This lower amount of antigen could explain why the sensitivity for their detection was low when compared to the other four genera.

Nonspecificity of general ELISA

A double-sandwich ELISA was used to test the ability of the mixed antibody to detect a wide variety of molds and yeasts. Reactivities of various molds and yeasts were compared to the standard prepared by mixing equal proportions of all six molds to a concentration of 0.1 mg/mL. All other molds and yeasts were tested at the 0.1 mg/mL concentration. Of the six genera injected into the rabbit to produce the mixed antibody, *Aspergillus*, *Fusarium*, and *Penicillium* species showed higher activities and could be detected more easily; however, various species within each of these genera showed different rates of detection (Table 1). The difference in detection may be due to the distinct immunodominant sites for the species. Some sites would be immunodominant, whereas, others would not. Research by Notermans and Heuvelman (1985) and Tsai and Cousin (1990) showed that not all species within a given genus reacted to the same degree with an antibody. Tsai and Cousin (1990) reported that antibodies to *A. versicolor* cross-reacted with antigens from species of *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Epicoccum* when only those antigens were used. However, the unrelated genera of *Alternaria*, *Fusarium*, and *Epicoccum* could not be detected in the presence of the specific antigen to which the antibody was produced. Similarly, antibodies to *M. circinelloides* showed cross-reactivity to antigens from species of *Mucor, Aspergillus*, and *Penicillium* but only to *Mucor* in competitive assays (Tsai and Cousin, 1990). Antibodies to *P. chrysogenum* cross-reacted with antigens from *Penicillium* and *Aspergillus* species. Antibodies to *C. herbarum* or *G. candidum* showed cross-reactivity only with antigens from species within its own genus (Tsai and Cousin, 1990). These results again suggested that the antibody should be made from the genus of interest if good reactivity with antigens was desired.

Table 1—ELISA cross-reactivities of the mixed mold antibody[a] with mycelial and cellular antigens from various molds and yeasts, respectively[b]

Fungi tested	Relative activity[c]
Alternaria species	++
Alternaria alternata	+
Alternaria cucumerina	++
Alternaria solani #1888	++
Aspergillus aluteus NRRL 407	++
Aspergillus candidus	+++
Aspergillus clavatus	++
Aspergillus fischeri NRRL 181	++
Aspergillus flavus ATCC 22546	++
Aspergillus flavus NRRL 13135	++
Aspergillus foetidus	+
Aspergillus fumigatus	+++
Aspergillus nidulans var. latus	++++
Aspergillus niger	+++
Aspergillus nomius NRRL 13137	++
Aspergillus ochraceus	++
Aspergillus oryzae NRRL 1988	++
Aspergillus oryzae NRRL 447	+++
Aspergillus parasiticus NRRL 13539	+++
Aspergillus parasiticus NRRL 3145	++
Aspergillus parasiticus NRRL 502	++
Aspergillus parasiticus NRRL 26691	++
Aspergillus petraki	++
Aspergillus repens ATCC 48521	+++
Aspergillus sojae NRRL 5594	++
Aspergillus tamarii NRRL 20818	++
Aspergillus terreus	++
Aspergillus versicolor	+++
Aspergillus wentii NRRL 377	+++
Aureobasidiuum pullulans ATCC 16622	++
Botrytis aclada #7465	++
Botrytis cinera #7432	++
Byssochlamys fulva 5-6	++++
Candida krusei	++
Candida lipolytica	+
Chaetomiium globosom NRRL 1669	++
Cladosporium species	++
Cladosporium herbarum ATCC 28987	++
Epicoccum nigrum ATCC 48208	++
Epicoccum rinomas	++
Erotium repens ATCC 42689	++++
Fusarium acuminatum #16560	+++
Fusarium avenaceum R8983	+++
Fusarium culmorum #1686	+++
Fusarium culmorum R-8545	+++
Fusarium graminearum	++++
Fusarium heterosporum #1336	++++
Fusarium moniliforme #2456	+++
Fusarium moniliforme #2460	+++
Fusarium moniliforme NRRL 13564	+++
Fusarium napiforme M-5909	+++
Fusarium nivale	+++
Fusarium nygamai M-6126	+++
Fusarium oxysporum #1904	+++
Fusarium oxysporum #2569	+++
Fusarium poae	+++
Fusarium proliferatum M-3446	+++
Fusarium roseum 'gibbosum' #564	+++
Fusarium sambucinum R-5463	+++
Fusarium semitectum R-3962	+++
Fusarium solani #2561	+++
Fusarium sporotrichioides	+++
Fusarium tricinctum T-429	+++
Geotrichum candidum NRRL Y-552	+
Hansenula anomola 67-455	+
Hansenula canadens 1-1252	+
Leptosphaerulina briosiana	+++
Monascus #1417	++++
Monascus ruber #1596	++++
Mucor species	++
Mucor Circinelloides NRRL 3614	+
Neosartorya fischeri #110483	+++
Penicillium aurantiogriseum 02-057-06	++++
Penicillium chrysogenum ATCC 10106	+++
Penicillium citrinum 46-006-05	+++
Penicillium corylophilum	+++
Penicillium crustosum	+++
Penicillium cyclopium	++++
Penicillium expansum	++++
Penicillium frequentans	++
Penicillium implicatum WDC 15	+++
Penicillium italicum	++
Penicillium lanosum	++++
Penicillium martensii NRRL 1843	++++
Penicillium miceyski 46-141-13	++++
Penicillium oxalicum NRRL 5202	+++
Penicillium spinulosum 46-104-08	+++
Penicillium urticae	++++
Penicillium varabile 02-072-16	+++
Penicillium viridicatum 02-057-09	+++
Phoma exigua #20763	+++
Pythium aphanidermatum	—
Rhizopus oligosporus	++
Saccharomyces cerevisiae (chablis)	—
Saccharomyces cerevisiae (champagne)	—
Schizophyllum commune	—
Sordaria fimicola	++
Six molds[b]	+++
Talaromyces flavus NRRL 13535	+++
Trichoderma species	++
Trichoderma #1219	++
Trichoderma viride GA-36	+++
Trichothecium roseum #2307	++++
Verticillium albo-atrum NRRL 1204	++++

[a] Mixed antibody produced from mycelia of six molds: *Aspergillus versicolor, Cladosporium herbarum, Fusarium poae, Geotrichum candidum, Mucor circinelloides,* and *Penicillium chrysogenum.*

[b] ELISA was done with 0.1 mg/mL of each mold or yeast antigen. Cross-reactivity was scored as follows: (−) A_{450} < Four Trial Values of Negative Control (NC); (+) Four Trial Values of NC < A_{450} < 20% Value of Positive Control (PC); (++) 20% Value of PC < A_{450} < 50% Value of PC; (+++) 50% Value of PC < A_{450} < 100% Value of PC; (++++) A_{450} > 100% Value of PC. PBS-Tween was the negative control and the mixture of six molds: *A. versicolor, C. herbarum, F. poae, G. candidum, M. circinelloides,* and *P. chrysogenum* were the positive controls.

[c] Relative activity = average ELISA reading of fungus tested/ELISA reading of antibody-producing mold in four trials.

Several molds gave >50% cross-reactivity for the mixed antibody, while others such as *Cladosporium*, *Geotrichum*, and *Mucor* species were detected with <50% cross-reactivity (Table 1), with relative activities lower than those of *Aspergillus*, *Fusarium*, and *Penicillium*. For *Cladosporium*, the low cross-reactivity may be due to not injecting enough antigen from *Cladosporium* because it grew more slowly than the other molds. Although it was injected at a similar concentration to the *Aspergillus*, *Fusarium* and *Penicillium*, a higher concentration or older age may be needed to produce a sensitivity similar to these molds. Genera that were not added to the mixture were detected, indicating several different immunodominant sites were recognized by many different genera of molds. This can be seen (Table 1) where species from genera such as *Byssochlamys*, *Eurotium*, *Leptosphaerulina*, *Monascus*, *Neosartorya*, *Phoma*, *Sordaria*, *Talaromyces*, *Trichoderma*, *Trichothecium*, and *Verticillium* showed high cross-reactivity although they were not inoculated into the mixture to produce the general ELISA. Results showed that one antibody could be used to detect several genera and many species within those genera. More research is needed on the best concentrations of various mold genera to develop a mixed antibody that could detect many different molds with higher cross-reactivity and greater nonspecificity.

Yeasts were essentially nondetectable (anything <50% would not be detected) with the antibodies (Table 1), suggesting that yeast cell walls had different immunogenic compositions. Therefore, yeast contamination would not interfere with mold detection by the general ELISA. Lin and Cousin (1987) and Tsai and Cousin (1990) also showed no cross-reactivity with yeasts.

Nonspecificity of the general ELISA to other molds

Mold genera other than those injected into the New Zealand white rabbits were used to determine the nonspecificity of the

mixed antibody to other molds when added to citrate buffer in concentrations 0 to 1 mg/mL. Experiments were done to determine if an increasing concentration of each individual mold could be detected in higher concentrations (Table 2). When comparing molds with a relative cross-reactivity of >0.5 (Table 1), they were detected in higher concentrations compared to molds with less relative cross-reactivity (Table 2). Molds such as *Alternaria species*, *A. pullulans*, *B. aclada* #17465, *E. nigrum* ATCC 48208, *R. oligosporus*, and *Trichoderma spp.* that had <0.5 relative cross-reactivity showed lower sensitivity and lower detectable antigen. One exception was *A. tamarii* NRRL 20818 that showed higher sensitivity at higher mold concentrations. Molds that showed a cross-reactivity of ≥0.5 (Table 1) generally had higher specificity and were detected in higher concentrations indicating that they may share common immunodominant sites with antibody-producing molds. Molds that were not used to produce the mixed antibody generally showed lower specificity in detection indicating that although several different molds were used, each genus had specific immunodominant sites. A mixed mold polyclonal antibody could detect several genera of mold antigens because they shared some common immunodominant sites, whether they were major (high cross-reactivity) or minor (low cross-reactivity). This ELISA method may be used to quantify molds if the relationship between dry weight and amount of mold could be determined.

Table 2—ELISA readings for the detection of increasing concentrations of selected molds diluted in citrate buffer using the mixed antibody[a]

	Mold concentration (μg/mL)[b]			
	10^0	10^1	10^2	10^3
Alternaria species	0.03	0.03	0.04	0.15
Aspergillus candidus	0.03	0.09	0.26	0.47
Aspergillus fumigatus	0.02	0.08	0.21	0.41
Aspergillus nidulans var. latus	0.13	0.29	0.47	0.63
Aspergillus niger	0.01	0.05	0.14	0.30
Aspergillus oryzae NRRL 447	0.01	0.04	0.12	0.20
Aspergillus parasiticus NRRL 13539	0.02	0.05	0.14	0.25
Aspergillus repens ATCC 48521	0.03	0.08	0.28	0.39
Aspergillus tamarii NRRL 20818	0.01	0.04	0.16	0.40
Aspergillus wentii NRRL 377	0.02	0.08	0.20	0.30
Aureobasidium pullulans	0.02	0.03	0.08	0.13
Botrytis aclada #7465	0.04	0.04	0.09	0.15
Byssoclamys fulva 5-6	0.10	0.22	0.36	0.44
Epicoccum nigrum ATCC 48208	0.01	0.03	0.06	0.15
Eurotium repens ATCC 42689	0.07	0.15	0.26	0.35
Fusarium acuminatum ATCC 16560	0.03	0.09	0.17	0.27
Fusarium graminearum	0.02	0.07	0.18	0.39
Leptosphaerulina bricsiana	0.00	0.02	0.06	0.20
Monascus species #1417	0.07	0.18	0.31	0.41
Monascus ruber #1597	0.07	0.16	0.33	0.44
Neosartorya fischeri #110483	0.09	0.14	0.20	0.30
Penicillium aurantiogriseum 02-057-06	0.01	0.04	0.17	0.39
Penicillium expansum	0.03	0.08	0.22	0.47
Penicillium urticae	0.03	0.05	0.16	0.35
Phoma exigua #20763	0.06	0.09	0.17	0.27
Rhizopus oligosporus	0.03	0.05	0.10	0.12
Sordaria fimicola	0.03	0.04	0.09	0.16
Talaromyces flavus NRRL 13535	0.09	0.14	0.19	0.27
Trichoderma species	0.01	0.04	0.07	0.16
Trichothecium roseum #2307	0.03	0.14	0.25	0.34
Verticillium albo-atrum NRRL 1204	0.03	0.09	0.14	0.22

[a] Antibody produced from mycelia of *Aspergillus versicolor, Cladosporium herbarum, Fusarium poae, Geotrichum candidum, Mucor circinelloides,* and *Penicillium chrysogenum.*

[b] Control values in buffer without the presence of molds ranging from 0.06 to 0.1 were subtracted from ELISA readings.

Detection by general ELISA of molds added to foods

For immunoassays to be used in the food industry, they must provide reproducible and consistent results with naturally contaminated and/or augmented food samples. When molds were added to dairy products, Tsai and Cousin (1990) showed low recoveries for some molds because casein interferred with the ELISA. In a comparison of several buffers (PBS-Tween, citrate, acetate, Tris-HCL), citrate buffer (pH 7.2) gave the best recovery of mold probably because it suspended casein and prevented it from binding to the microtiter plate and interferring with the antigen-antibody reaction. When citrate buffer (pH 7.2) was used to analyze yogurt, cottage and Cheddar cheeses that had added to it 0 to 1 mg/mL of a mixture of the six freeze-dried molds, *A. versicolor*, *C. herbarum*, *F. poae*, *G. candidum*, *M. circinelloides*, and *P. chrysogenum* were detected at 1 μg/mL (Fig. 2). Molds were detected better in citrate buffer than in dairy products when concentrations were >100 μg/mL, but at very low mold concentrations there were no differences in detection. Since dairy products with 100 μg/mL of molds are highly contaminated, such a difference may not be a concern in normal detection.

When other commercial products such as fruit juices, raisins, and nonfat dry milk were augmented with molds other than the six used to produce the mixed antibody, detection was also observed with an increase in ELISA readings as mold concentration increased (Fig. 3). Furthermore, commercial products such as various types of cheeses, (medium Cheddar, Mozzarella, and Wisconsin cheeses), and various types of spices, (nutmeg, white pepper, paprika, and cinnamon) were analyzed using the general ELISA. Initial readings were high for all spices and cheeses at an initial 10-fold dilution indicating that those products were previously contaminated by molds, and that such common molds could be detected by the mixed antibody. When the spices had molds added, there were no differences in ELISA readings before and after mold addition because they were already contaminated by mold antigens at a level beyond detection. Molds are common contaminants of spices, and the antigens can remain after decontamination.

Detection of molds inoculated into dairy products

Once the presence of added mold could be detected, it was then essential to determine if mold growth could be monitored. Therefore, viable molds were added to cottage cheese and yogurt to determine how soon an increase in mold contamination could be detected in dairy products. As shown (Tables 3–4), a mixture of four molds; (*A. versicolor*, *C. herbarum*, *F. poae*, and *P. chrysogenum*) could be detected in cottage cheese and yogurt incubated at 22°C for 10 days and at 7°C for 22 days. *G. candidum* and *M. circinelloides* were not inoculated into these products because they grew too fast and outgrew the other molds. At 22°C, the four molds grew very quickly in both the cottage cheese and the yogurt and could be detected as early as day 2 before visualization was possible, but at 7°C, the 4 molds grew slower at the lower temperature (Table 3). When plate counts were compared to ELISA for all four inoculation experiments, the increase in plate count readings paralleled increases in ELISA (Tables 3–4). Therefore, the ELISA had the potential to be used in place of the standard plate count to determine total mold contamination.

It is not possible to give an ELISA reading that will indicate a specific plate count because there are many variables involved in the plate count method. These include dilutions, necessity to grow mold on defined media, temperature of growth, etc. ELISA detects the presence of mold cell wall components regardless of conditions. Notermans et al. (1988a) found that the comparison between the mold colony count method and the ELISA could not be made for enumeration of molds in nuts and spices because they did not have actively growing molds. If nonviable molds were present, then their antigens could also be detected by the ELISA.

However, in our research, molds were grown in food samples and analyzed for growth by colony-count and ELISA. Four molds were inoculated into cottage cheese and yogurt and all four molds were present for the first 6 days after inoculation. After day 10, *A. versicolor* and *P. chrysogenum* became dominating in the population at 7°C. However, by day 4 at 22°C,

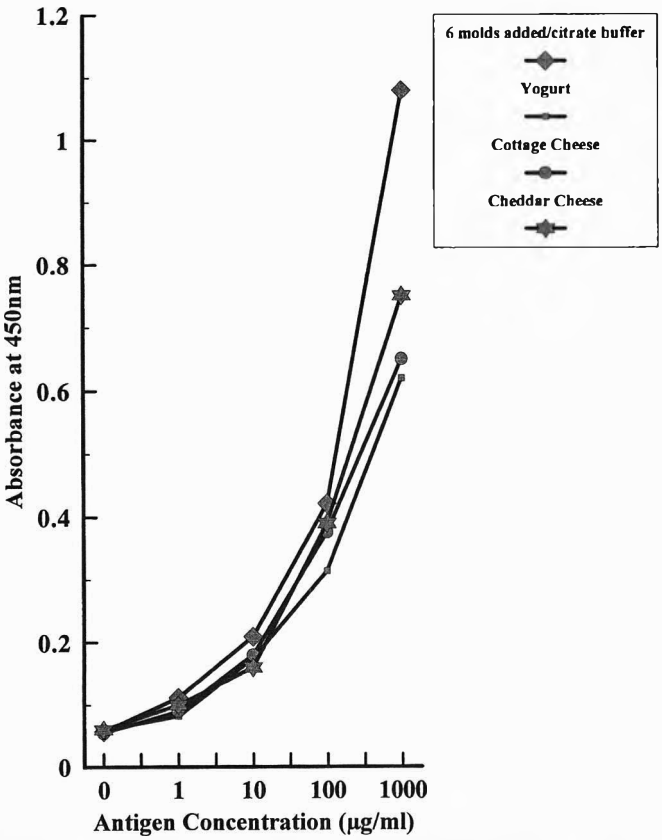

Fig. 2—ELISA for six molds (*A. versicolor, C. herbarum, F. poae, G. candidum, M. circinelloides,* and *P. chrysogenum*) added to citrate buffer and dairy products.

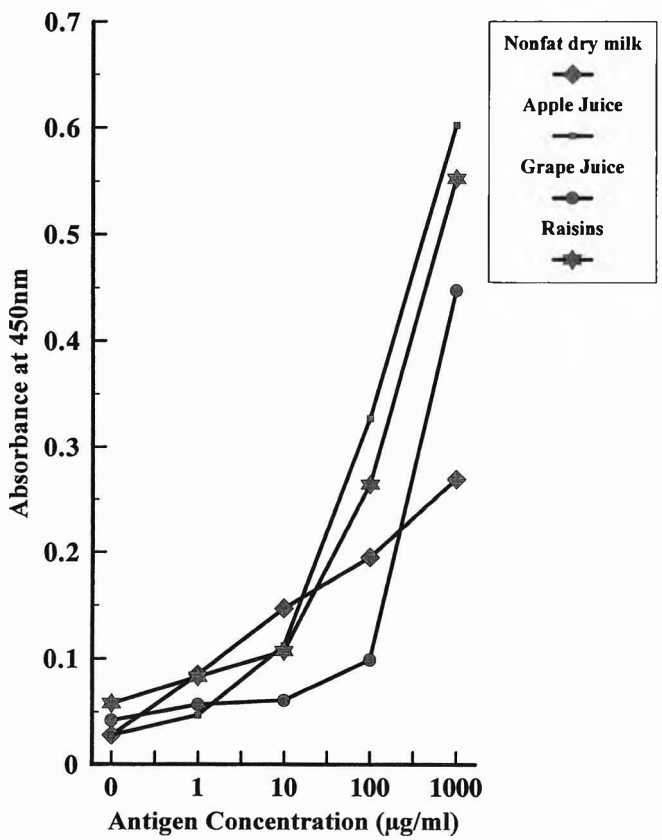

Fig. 3—ELISA for molds added to apple juice (*Byssochlamys fulva*), grape juice (*Botrytis cinerea*), raisins (*Aspergillus candidans*), and nonfat dry milk (*Fusarium poae*).

Table 3—Colony counts and ELISA readings for cottage cheese and yogurt inoculated with a mixture of four molds, *A. versicolor, C. herbarum, F. poae,* and *P. chrysogenum,* and incubated at 22°C for 10 days

	Cottage cheese		Yogurt	
Day	Plate count (CFU/g)[a]	ELISA[b]	Plate count (CFU/g)[a]	ELISA[b]
0	6.9×10^2	0.01	6.8×10^2	0.00
2	3.4×10^3	0.04	1.3×10^3	0.04
4	1.7×10^5	0.36	6.2×10^5	0.11
6	2.2×10^6	0.53	5.6×10^6	0.12
8	3.3×10^6	0.74	3.0×10^6	0.14
10	2.5×10^6	0.59	2.7×10^6	0.21

[a] All controls were estimated as < 70 colony forming units/gram (cfu/g) because there were no colonies on the plates.
[b] Control values ranging from 0.03 to 0.06 were subtracted from the ELISA readings.

Table 4—Colony counts and ELISA readings for cottage cheese and yogurt inoculated with a mixture of four molds, *A. versicolor, C. herbarum, F. Poae,* and *P. chrysogenum,* and incubated at 7°C for 22 days

	Cottage cheese		Yogurt	
Day	Plate Count (CFU/g)[a]	ELISA[b]	Plate Count (CFU/g)[a]	ELISA[b]
0	1.3×10^3	0.01	1.3×10^3	0.03
4	1.3×10^3	0.01	2.8×10^3	0.03
6	2.5×10^3	0.02	2.9×10^3	0.12
10	2.7×10^3	0.05	3.0×10^3	0.13
14	3.1×10^3	0.28	3.3×10^3	0.22
18	7.2×10^3	0.38	4.3×10^3	0.34
22	1.2×10^4	0.46	7.3×10^4	0.35

[a] All controls were estimated as < 70 colony forming units/gram (cfu/g) because there were no colonies on the plates.
[b] Control values ranging from 0.03 to 0.08 were subtracted from the ELISA readings.

these two molds plus *C. herbarum* were dominant in yogurt (Table 4). This may explain why there was a relatively higher ELISA reading in samples with *A. versicolor* and *P. chrysogenum* compared to those with the *C. herbarum* because it had a lower sensitivity in the assay. The plate count could not be compared directly to ELISA because the difference in mold growth could lead to differences in detection based on sensitivity.

This research supports earlier work done by Tsai and Cousin (1990) who inoculated yogurt and Cheddar and cottage cheeses individually with *A. versicolor, C. herbarum, G. candidum, M. circinelloides,* and *P. chrysogenum* and incubated them at 5 and 22°C. ELISA readings increased by day 4 at 5°C and by day 2 for 22°C and continued to increase for 20 days. The ELISA could detect the early growth of mold before it could be seen on the products.

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Ms received 3/29/95; revised 6/23/95; accepted 7/9/95.

Journal Paper No. 14604 of the Purdue Agricultural Experiment Station.
We thank the National Dairy Research and Promotion Board for partially supporting this research and Dr. L.R. Beuchat, L.B. Bullerman, H. Gourama, P.E. Nelson, P.V. Nielsen, and S.W. Peterson for supplying fungal cultures. The technical assistance of Jennifer Moseley is gratefully acknowledged.

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Ms received 1/17/95; revised 6/10/95; accepted 7/6/95.

Antibacterial Activity of Carvacrol, Citral, and Geraniol against *Salmonella typhimurium* in Culture Medium and on Fish Cubes

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ABSTRACT

Carvacrol, citral and geraniol showed potent antibacterial activity against *Salmonella typhimurium* and its rifampicin-resistant (Rif^R) strain as determined in tryptic soy broth and by zone of inhibition on agar-based medium. Carvacrol had the most potent bactericidal activity, with minimum inhibitory and bactericidal concentrations (MIC and MBC) of 250 µg/mL for both tester strains. When tested at 0.5, 1.5 and 3.0% in 1% Tween 20 for bactericidal activity against Rif^R-*S. typhimurium* inoculated on fish cubes, carvacrol at 3.0% completely killed the inoculated bacteria, while geraniol killed most of the bacteria, and citral killed the least. Carvacrol and geraniol showed potent antibacterial activity at 1.5%. Bactericidal activity became more evident as storage of fish cubes at 4°C lengthened. The comparable inhibition of these strains of *Salmonella* and species of Gram-negative bacteria by carvacrol and geraniol support their application as potential antibacterial agents in food systems.

Key Words: fish, salmonella, carvacrol, citral, geraniol

INTRODUCTION

FOODBORNE ILLNESS resulting from consumption of contaminated foods with pathogenic bacteria and/or their toxins has been of great concern to public health. Bacterial pathogens are estimated by U.S. Department of Agriculture staff to be responsible for 3.6 to 7.1 million foodborne disease cases each year, with associated costs and productivity losses of $2.9 to $6.7 billion (Morris, 1995). Controlling pathogenic microorganisms would reduce foodborne outbreaks and assure consumers a continuing safe, wholesome, and nutritious food supply.

Antimicrobial agents and organic acids have been used to inhibit growth of foodborne bacteria and to extend the shelf-life of processed foods. Many naturally occurring phenolic compounds, including coumarins, flavonoids and essential oils, found in edible and medicinal plants, herbs, and spices, have shown antimicrobial functions and could become new antibacterial agents (Deans and Svoboda, 1988; Janssen et al., 1985; Kubo et al., 1992). The antimicrobial activity of spices and essential oils is well recognized; essential oils of thyme, cinnamon, bay and clove possess antimicrobial activity (Shelef et al., 1980; Deans and Ritchie, 1987; Farag et al., 1989). Essential oils and their constituents have been used extensively as flavor ingredients in a wide variety of food, beverage, and confectionery products. Many such compounds are classified as Generally Recognized As Safe.

Kim et al. (1995) studied the antibacterial activity of 11 essential oil constituents in culture medium against five foodborne pathogens, *Escherichia coli, E. coli* O157:H7, *Salmonella typhimurium, Listeria monocytogenes*, and *Vibrio vulnificus*, and demonstrated that carvacrol [2-methyl-5-(1-methylethyl)phenol], citral (3,7-dimethyl-2,6-octadienal), and geraniol (3,7-dimethyl-2,6-octadien-1-ol) had potent bactericidal activity. In meat products, especially beef, poultry and seafood, *E. coli, E. coli* O157:H7, *S. typhimurium, L. monocytogenes*, and *V. vulnificus* are common pathogens responsible for foodborne outbreaks from such products. In addition to developing rapid methods for determining pathogens in foods, it is also important to find treatments that can mitigate or eradicate them. Our objective was to examine the bactericidal effectiveness of three essential oil compounds against *S. typhimurium* in culture medium and on fish cubes. A rifampicin-resistant *S. typhimurium* was used to facilitate recovery and distinguish itself from naturally occurring microflora.

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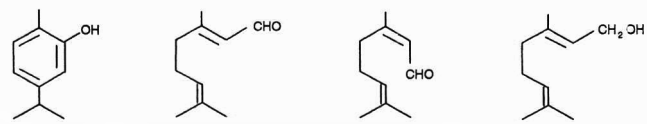

Fig. 1—Structures of carvacrol, citral, and geraniol.

MATERIALS & METHODS

Test compounds

Carvacrol and citral (Fig. 1) were purchased from Aldrich Chemicals (Milwaukee, WI), and geraniol (Fig. 1) was obtained from Sigma Chemical Company (St. Louis, MO). The compounds were checked for purity by thin-layer chromatography and used without further purification. Solutions containing the desired concentrations of test compounds were freshly prepared before each use by dissolving them in solution of 0.1g Tween 20 (Fisher Scientific, Orlando, FL) in 10 mL sterile distilled water.

Test microorganisms

Salmonella typhimurium (ATCC 6539) and its rifampicin-resistant (Rif^R) strain were maintained on tryptic soy agar (TSA, Difco Laboratories, MI) plates. They were transferred to fresh tryptic soy broth (TSB, Difco) before use. The Rif^R strain of *S. typhimurium* was isolated by streaking a *S. typhimurium* (ATCC 6539, parental strain) colony from a TSA plate onto a RIF-TSA plate. RIF-TSA plates were prepared by adding 0.1g rifampicin (Sigma) dissolved in 5 mL methanol to 1L of sterilized TSA. The stability of this rifampicin-resistant phenotype was verified by inoculating a single colony of Rif^R-*S. typhimurium* from a RIF-TSA plate into TSB and incubating at 35°C for 16 hr with shaking at 140 rpm. The bacterial suspension was streaked on RIF-TSA plates and incubated at 35°C for 24 hr. After the isolated colony was picked, inoculated in TSB, and incubated at 35°C for 8 hr with shaking, the bacterial suspension was again streaked on RIF-TSA plates. This same

Table 1—Zone of inhibition of citral, carvacrol, geraniol against *Salmonella typhimurium* and its rifampicin-resistant strain

	Conc.	*S. typhimurium*	Rif^R-*S. typhimurium*
Citral	5%	0.86 ± 0.02^{Aa}	0.84 ± 0.04^A
	10%	0.93 ± 0.08^A	1.00 ± 0.03^B
	15%	1.07 ± 0.09^B	1.08 ± 0.05^C
	20%	1.12 ± 0.14^B	1.12 ± 0.07^C
Carvacrol[b]	5%	1.37 ± 0.05^A	1.63 ± 0.06^A
	10%	1.89 ± 0.08^B	2.09 ± 0.24^B
Geraniol	5%	0.92 ± 0.06^A	1.03 ± 0.05^A
	10%	1.05 ± 0.11^B	1.14 ± 0.08^B
	15%	1.11 ± 0.13^B	1.27 ± 0.11^C
	20%	1.33 ± 0.13^C	1.29 ± 0.06^C

[a] Zone of inhibition was expressed as diameter in cm. Values represent mean diameter ± standard deviation of inhibition zone from four experiments, each using quadruplicate plates. Within each column and for each compound, means with same superscripts (A, B, and C) were not significantly different.

[b] Carvacrol was dissolved in 2% Tween 20. This compound at 15 and 20% did not dissolve in 2% Tween 20 and was therefore not tested.

The control, 1 and 2% Tween 20, showed no zone of inhibition.

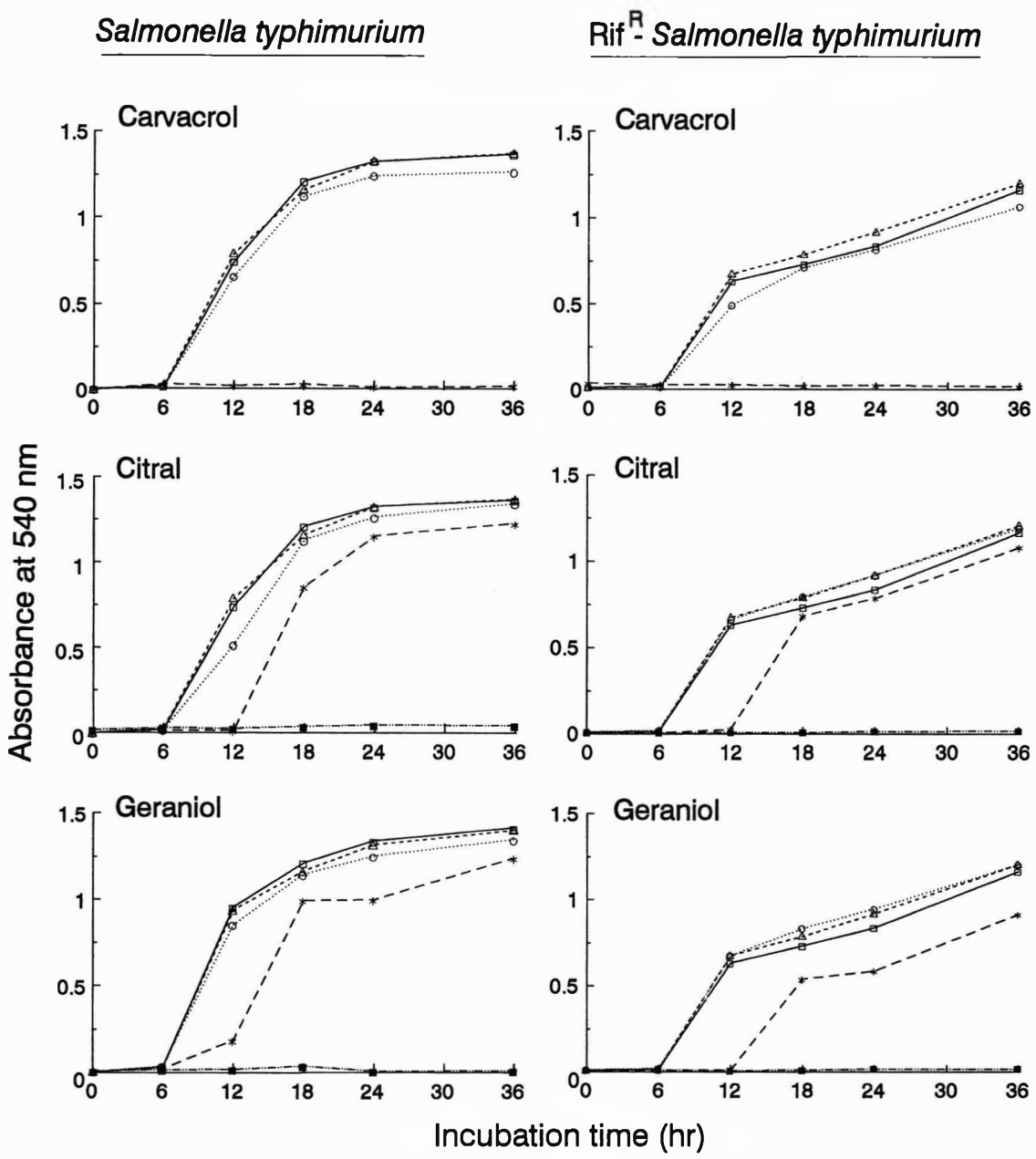

Fig. 2—Time-related growth of *Salmonella typhimurium* and its rifampicin-resistant strain in tryptic soy broth in the presence of carvacrol, citral, and geraniol. —□— buffer, —△— Tween 20, —○— 100 ppm, —*— 250 ppm, and —■— 500 ppm.

process was repeated two more times, and the Rif^R-*S. typhimurium* isolate was maintained on TSA.

Preparation of red grouper (*Epinephelus morio*) cubes

Red grouper fillets were obtained from a local seafood store in Gainesville, FL. After storage at −20°C for 2 hr, the fillets were cut into cubes (about 2.5 × 2.5 × 2.5 cm). Fish cubes were packed in plastic bags and stored at −20°C. One day before use, the cubes were thawed in a 4°C cold room.

Antibacterial assay using a paper disc method

The three essential oil constituents were tested on *S. typhimurium* and its Rif^R-strain using a zone of inhibition assay on TSA. Bacterial number of a 16-hr culture in TSB was estimated by comparing the absorbance at 540 nm, using a Beckman DU-40 spectrophotometer (Fullerton, CA), after diluting with TSB and calibrating to a standard curve. The concentration was then adjusted to 4×10^8 colony forming units (CFU)/mL using Butterfield's phosphate buffer (pH 7.2), and a 100 µL aliquot was evenly spread on TSA plates using a glass rod spreader. The plates were left at room temperature for 20 min to allow the agar surface to dry. Sterilized filter paper discs (Whatman No. 1 filter paper, 0.6 cm in diameter) were arranged on the plate, and a 10 µL aliquot of test solutions in 1% Tween 20 was added to the paper discs. Five discs were arranged on a plate; two each for 5 and 20% concentrations of the test chemical, and one for the control (or two each for 10 and 15% concentrations of the test chemical, and one for control). After the plates were incubated at 35°C for 24 hr, diameters of distinctly clear zones were measured using a metric ruler with the aid of a Darkfield Quebec colony counter (American Optical Co., Buffalo, NY). Four discs were used for each test compound at each concentration. Chloramphenicol was also included as positive control. The experiment was repeated four times.

Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC)

The MIC of test compounds was determined using the broth dilution method. To each duplicate 50 mL Erlenmeyer flask containing 19.6 mL of sterile TSB was added a 200 µL aliquot of bacterial suspension at 10^6 CFU/mL. Test compounds were then added in 200 µL to give final concentrations of 100, 250, 500, or 1000 µg/mL. Flasks were incubated

Table 2—Effect of carvacrol, citral, and geraniol on the growth of rifampicin-resistant *Salmonella typhimurium* in fish stored at 4°C

	Concentration % (w/v)	Log (CFU/g + 1) on sampling days			
		0	1	2	4
Tween20[a]		6.13 ± 0.18	5.92 ± 0.41	4.89 ± 0.39	4.24 ± 0.67
Buffer[a]		6.12 ± 0.19	5.59 ± 0.48	4.59 ± 0.52	3.69 ± 0.40
Carvacrol[b]	0.5	5.39	4.25	3.65	2.97
	1.5	1.84	1.11	0.83	0.31
	3.0	0	0	0	0
Citral[b]	0.5	5.68	5.01	4.45	3.65
	1.5	5.38	4.04	3.05	1.52
	3.0	4.55	2.08	1.22	0.62
Geraniol[b]	0.5	5.63	4.22	3.30	2.55
	1.5	4.83	2.29	1.33	0.96
	3.0	0.51	0.55	0.41	0.73

[a] For Tween 20 and buffer, data are average ± standard deviation of 12 readings (quadruplicate samples and three trials).

[b] For carvacrol and citral, data are averages of 12 readings (quadruplicate samples and three trials). For geraniol, data are averages of 10 readings.

with shaking at 140 rpm in a 35°C water bath. At 0, 6, 12, 18, 24 and 36 hr, a 1.0 mL aliquot was removed from each flask and turbidity at 540 nm was measured. Duplicate flasks were treated for each compound at each concentration. The lowest concentration at which no growth occurred in either flask was taken as MIC. After determining MIC, the flask showing no increase in turbidity at each time interval (0, 6, 12, 18, 24, and 36 hr) was streaked on TSA plates to check bacterial growth/survival. The MBC was the lowest concentration at which the test compound killed all of the bacteria. This experiment was conducted twice with duplicate samples for each compound at each test concentration.

Treatment of Rif^R-*S. typhimurium* inoculated fish cubes with carvacrol, citral, and geraniol

Fish cubes (4.4 kg) were mixed by stirring for 5 min at 25°C with 4,400 mL of Rif^R-*S. typhimurium* suspension at 10^7 CFU/mL in Butterfield's phosphate buffer. After the extra liquid was drained off in a metal container, the cubes were weighed and divided into eleven 400-g portions. Each portion was then dipped for 10 min in 400 mL of Butterfield's phosphate buffer (control), 1% Tween 20 (control), or carvacrol, citral, or geraniol at 0.5, 1.5, and 3.0% (w/v) in 1% sterile Tween 20 in a 1-L flask. After draining off extra liquid from the samples, 25-g subsamples were placed in sterile 6.3 × 10 cm Whirl-pak™ bags (Nasco, Fort Atkinson, WI), and then stored at 4°C for 4 days. Four replications were used for each test condition on each day. The experiment was repeated three times.

At each storage period (SP) (0, 1, 2 and 4 days), quadruplicate samples were randomly removed from each treatment group and microbial counts determined. The 25-g portion of the fish sample was homogenized with 225 mL Butterfield's phosphate buffer in a blender for 1 min. After serial dilution of the homogenate with Butterfield's phosphate buffer, 0.1 mL aliquots of each dilution were surface plated on four RIF-TSA plates. In some cases, a pour plate method was employed using 1 mL of the homogenate. The plates were incubated at 35°C for 48 hr and colonies were counted.

Sensory attributes (appearance and odor) of treated fish cubes were also examined informally by the authors.

Statistical analysis

In the antibacterial assay study, data from each of the three chemicals were analyzed for each bacterial strain separately by analysis of variance using PROC GLM of the Statistical Analysis System (SAS Institute, 1982). Differences between average zones of inhibition values for the different concentrations of each chemical were determined by Waller-Duncan k-ratio test (Waller and Duncan, 1969).

The bactericidal effects of citral, carvacrol, and geraniol on Rif^R-*S. typhimurium* inoculated fish cubes were investigated across concentrations and periods of storage using analysis of variance. Comparisons were made between treatment types (buffer, Tween 20, carvacrol, citral, and geraniol), chemical concentrations (0.5, 1.5, and 3.0%), SP (0, 1, 2, and 4 days), as well as all interactions between them. Bacterial numbers for the fish cube experiment were transformed to $\log_{10}$ (CFU/g + 1) as a variance stabilizing measure. The 3-way interaction of chemical type by concentration by SP was highly significant ($P < 0.01$), leading us to investigate the effects of chemical concentration and SP for each chemical separately. These effects were measured by fitting multiple regression equations using PROC REG of SAS Institute, Inc. (1982).

Initially, a second-degree regression equation was fitted as $\log_{10}$ (CFU/g + 1) = $\beta_0 + \beta_1$ Con. + β_2SP + β_3Con. × SP + β_4Con.^{2} + β_5SP^2 where the terms β_1Con. and β_2SP represent the linear effects of concentration and SP on $\log_{10}$ (CFU/g + 1); β_3Con. × SP represents a linear by linear interaction effect between concentration and SP, and β_4Con.^{2} and β_5SP^2 represent the curvilinear (quadratic) effects of concentration and SP on $\log_{10}$ (CFU/g + 1), respectively. Upon fitting and testing the significance of the terms in the model, second-degree terms that did not attain a significance level of $P \leq 0.05$ were dropped and the model refitted to produce a simpler, reduced model form. Plots of the estimated $\log_{10}$ (CFU/g + 1) surfaces for carvacrol and citral were generated using the CONTOUR function in the S language (Becker et al., 1988).

RESULTS & DISCUSSION

RIFAMPICIN-RESISTANT (RIF^R) STRAIN of *S. typhimurium* was used to facilitate differentiation of inoculated bacteria from natural flora. The Rif^R strain had a very similar growth pattern in TSB as the parent strain, ATCC 6539 (data not shown). The rifampicin-resistant phenotype was stable as demonstrated by consistent colony numbers of the culture diluents on TSA and RIF-TSA plates; no difference in colony numbers occurred with the two types of agar plates.

Antibacterial activity determined by paper disc and liquid culture assays

Carvacrol, citral, and geraniol showed dose-related inhibitions against *S. typhimurium* and its Rif^R strain using the paper disc assay (Table 1). In general, the two tester strains responded similarly to the test compounds. For each test compound with each tester strain, the zone of inhibition was different ($P < 0.05$) for the lower and higher concentrations (Table 1). Chloramphenicol at 0.25–2 µg/disc was included as the positive control; it gave a clear zone of inhibition with a diameter of 1.16–2.48 cm with Rif^R-*S. typhimurium*. Tween 20 was used to increase the solubility of hydrophobic compounds and aid in their penetration into bacterial cell walls and membranes. Overall, carvacrol was the most potent inhibitor, followed by geraniol, and citral. Growth patterns of *S. typhimurium* and its Rif^R strain in TSB in the presence of citral, carvacrol, or geraniol were compared (Fig. 2). The growth of both tester strains was completely inhibited by carvacrol at 250 µg/mL, and by citral and geraniol at 500 µg/mL. Streaking of test samples on TSA did not demonstrate bacterial growth. Therefore, carvacrol had a MIC and MBC value < 250 µg/mL against both tester strains, and citral and geraniol each had a MIC and MBC value < 500 µg/mL. Citral and geraniol at 250 µg/mL delayed the lag phase of the growth curve of both tester strains (Fig. 2). These compounds at 250 µg/mL might have killed some of the initial bacterial populations, and then affected the cellular structures or biochemical reactions of the growing bacterial cells. Once the bacteria overcame the inhibitory effect, they multiplied rapidly. Again, carvacrol was demonstrated in liquid culture assay to be a more potent antibacterial agent than geraniol and citral.

The relative antibacterial activity of carvacrol, geraniol, and citral against four other foodborne pathogens *Escherichia coli, E. coli* O157:H7, *Listeria monocytogenes*, and *Vibrio vulnificus* has been demonstrated by Kim et al. (1995). Antibactericidal activity of these compounds against *S. typhimurium* and its Rif^R strain as determined by the paper disc assay correlated with that in a liquid culture assay. Antibacterial agents with a low bacteriostatic/bactericidal activity against a tester strain would have a high MIC/MBC, and produce only a small zone of inhibition or no zone on agar plates.

Carvacrol, geraniol, and citral apparently had different modes of action against the *Salmonella* tester strains. Essential oil compounds exert antibacterial activity by (1) interfering with the phospholipid bilayer of the cell membrane, causing increased permeability and loss of cellular constituents (Knobloch et al., 1986); (2) impairing a variety of enzyme systems, including those involved in the production of cellular energy and synthesis

Table 3—Coefficient estimates and coefficient of determination values (R^2) for final fitted regression equations $\log_{10}$ (CFU/g + 1) = β_0 + β_1 Con. + β_2SP + β_3 Con. × SP + β_4 Con.^{2} + β_5SP^2 for each chemical

	Coefficient estimates						
	β_0	β_1	β_2	β_3	β_4	β_5	R^2
Citral	6.8652	−1.0218	−1.5295			0.1812	0.6653
Carvacrol	7.8537	−5.4503	−1.3986	0.4687	0.9443	0.1696	0.8568
w/o 3.0%	7.0040	−3.4196	−1.1413	0.2152		0.1108	0.7723
Geraniol	6.7555	−1.8625	−1.9427	0.3170		0.2175	0.8158

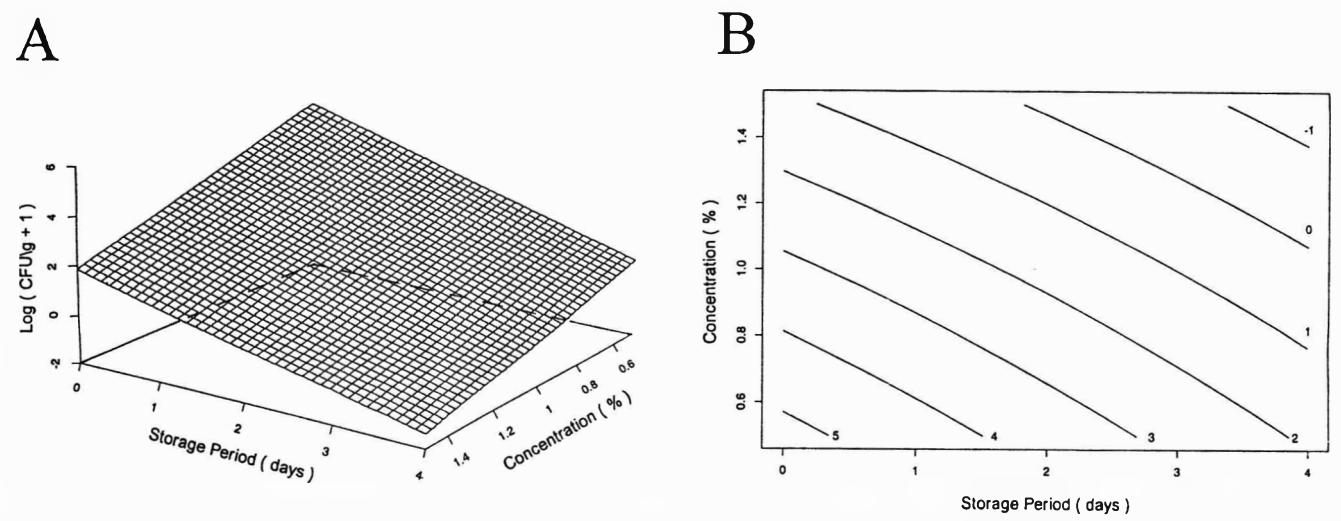

Fig. 3—Three-dimensional (A) and contour plots (B) of $\log_{10}$ (CFU/g + 1) showing relationships of carvacrol concentration and storage period. Only data from carvacrol at 0.5 and 1.5% were included. Contour lines (B) represent estimated $\log_{10}$ (CFU/g + 1) at various combinations of concentration and storage period.

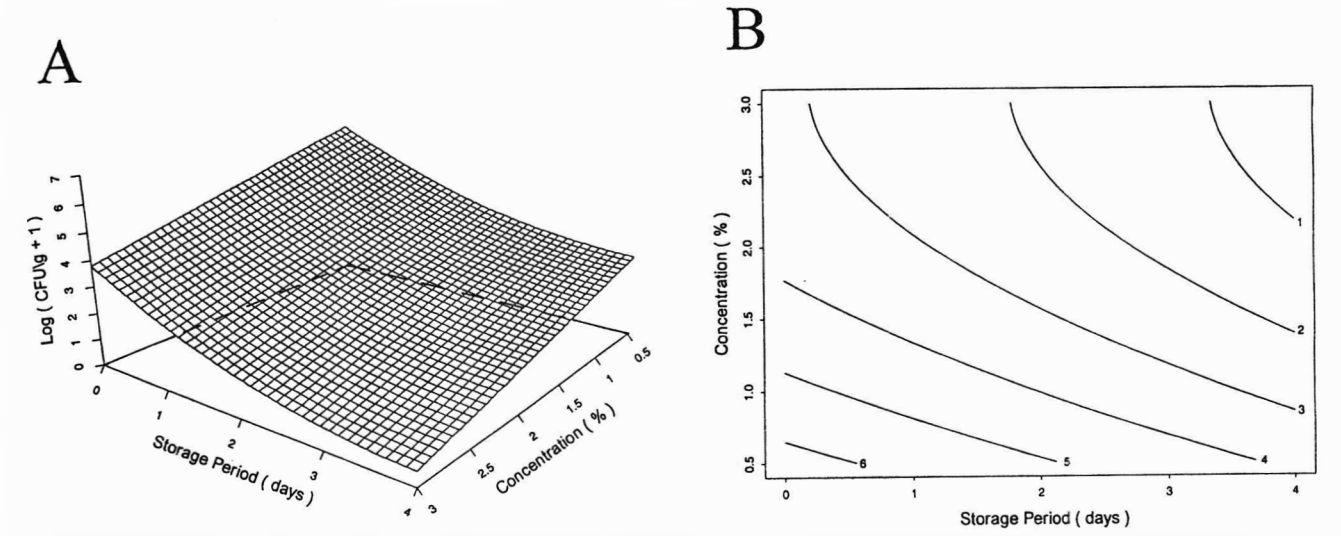

Fig. 4—Three-dimensional (A) and contour plots (B) of $\log_{10}$ (CFU/g + 1) showing relationships of citral concentration and storage period. Contour lines (B) represent estimated $\log_{10}$ (CFU/g + 1) at various combinations of concentration and storage period.

of structural components (Conner and Beuchat, 1984); and/or (3) inactivating or destroying genetic material.

Effect of carvacrol, citral, and geraniol on Rif^R-*S. typhimurium* inoculated on fish cubes

Time-related survival of Rif^R-*S. typhimurium* on inoculated fish cubes was compared (Table 2) following treatment with carvacrol, citral, and geraniol at 0.5, 1.5 and 3.0% in 1% Tween 20. Data from each of the three trials showed consistent patterns, so the data were pooled and averaged to illustrate overall effects of chemical treatments. Bacterial numbers in control groups (Butterfield's buffer and 1% Tween 20) decreased as periods of storage (4°C) of fish cubes increased. Fish samples treated with carvacrol, citral, and geraniol showed higher bactericidal effects. The test compounds also showed a dose-related bactericidal activity; carvacrol at 3% completely (100%) killed inoculated bacteria on day 0, and geraniol at the same concentration almost completely (>99%) killed all bacteria. A comparison among the compounds showed that carvacrol was the most potent bactericidal agent, followed by geraniol, and citral. Both control and treated fish cubes showed no signs of spoilage during 4-days storage at 4°C.

For each of the test chemicals, estimates of regression coefficients that were significant ($P < 0.05$) in the final fitted model are listed (Table 3). The criteria for determining best fit of the

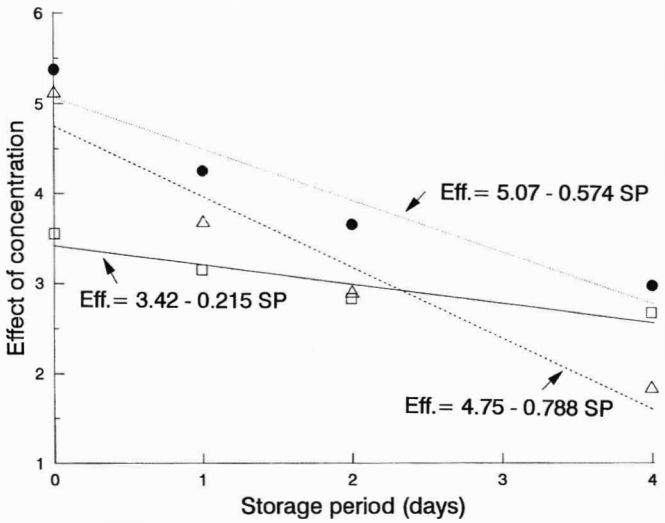

Fig. 5—Bactericidal effects of carvacrol and geraniol concentrations as related to storage time of inoculated fish cubes. —□— with carvacrol, the effect was defined as the difference in averages of $\log_{10}$ (CFU/g + 1) at 0.5 and 1.5%; —●— with carvacrol, the effect was defined as the difference in averages of $\log_{10}$ (CFU/g + 1) at 0.5 and 3.0%; and —△— with geraniol, the effect was defined as the difference in averages of $\log_{10}$ (CFU/g + 1) at 0.5 and 3.0%.

models were residual patterns, value of the coefficient of determination (R^2), and knowledge of the usual physical behavior of the system.

For carvacrol and citral, the surface shapes (Fig. 3 and 4) generated by the final model forms illustrate the effects of chemical concentration and SP on bacterial counts as $\log_{10}$ (CFU/g + 1). For all chemicals, the effect of concentration was linear with the log count at the higher concentration (carvacrol at 1.5%, citral and geraniol at 3.0%) lower ($P < 0.01$) than that at the lower concentration (each chemical at 0.5%). The effect of SP was curvilinear with greater reductions in log counts following treatment between 0 to 2 days than between 2 to 4 days. For both carvacrol and geraniol, the reduction in log counts with increasing concentration was greater at the 0 to 1 day period than at the 2 to 4 day period. The three-dimensional surface plots clearly show linear (Fig. 3A) and curvilinear (Fig. 4A) trends in log counts across levels of concentration and storage period. The contour plots (Fig. 3B and 4B) on the other hand can help estimate or predict values of log count for various combinations of concentration and storage time. For example (Fig. 3B), the curve for the value $\log_{10}$ (CFU/g + 1) = 2 defines all combinations of carvacrol concentration and storage period estimated to produce a bacterial count of 99, including combinations that were not used in the experiment.

At each day of storage, the concentration effect is defined as the difference between the average $\log_{10}$ (CFU/g + 1) at 0.5% and at 1.5% or 3.0% with carvacrol and at 0.5% and at 3.0% with geraniol (using averages from Table 2). A plot of these differences or effects, showed how their magnititudes decreased with increased storage period (Fig. 5). Simple linear regression equations of the effects of concentration Vs storage period are: estimated concentration effect = 3.42 − 0.215 SP for carvacrol, and estimated concentration effect = 4.75 − 0.788 SP for geraniol (Fig. 5). Both slope estimates were different ($P < 0.05$) from zero. However, but because the effects of concentration with carvacrol were based on only a 1% (0.5–1.5%) increase while the effects of geraniol were based on a 2.5% (0.5–3.0%) increase, a comparison of slopes of the two lines would not be valid. When the effect of carvacrol concentration was defined as the difference in average $\log_{10}$ (CFU/g + 1) at 0.5% and 3.0%, resulting in the equation, estimated concentration effect = 5.07 − 0.574 SP, the slope (−0.574) of this line did not differ ($P > 0.05$) from the slope (−0.788) of the geraniol line (Fig. 5). Thus, over a storage period of 0 to 4 days, decreases in the effects of concentration, as measured between 0.5 and 3.0% for carvacrol and geraniol, were not different.

Our results confirmed that *S. typhimurium* could survive on fish cubes at refrigerated temperatures. Food matrix composition, pH, and bacterial attachment on food systems affect the survival/growth of *S. typhimurium* on fish cubes at refrigerated temperatures. D'Aoust et al. (1982) studied the toxicity of surfactants used to facilitate the detection of *S. typhimurium* in naturally contaminated fatty food and showed that Tween 20 at 10% (w/v) was not toxic to *S. typhimurium*. Paper disc and liquid culture assays confirmed that 1% Tween 20 was not toxic to *S. typhimurium* and its Rif^R strain.

Treatment of inoculated fish cubes with carvacrol, citral, and geraniol may injure bacterial structures so that storage at refrigerated temperatures synergistically enhanced the bactericidal effect. Bacteria are, in general, sensitive to sudden changes in temperature (Traci and Duncan, 1974). This could account for the more effective bactericidal activities of these compounds against Rif^R-*S. typhimurium* inoculated on fish cubes.

The use of 0.1% carvacrol in 1% Tween 20 did not kill *S. typhimurium* inoculated on fish samples in preliminary studies. The reduction of antimicrobial activity in fish samples could be due to interactions of these essential oils with food components, such as proteins and lipids, which may have decreased levels of the compounds available for bactericidal functions. However, a much higher concentration (1.5%) was needed for each test compound to effectively kill this bacterium. Fish lipids may also affect the activity of these essential oils due to hydrophobic properties of the compounds. Unlike liquid media such as TSB, bacteria inoculated onto fish cubes could attach firmly to fish flesh resulting in reduced exposure to essential oils.

We noted that carvacrol gave a warmly pungent flavor to treated fish samples. Those fish cubes treated with citral had a yellowish color with a lemon-like flavor. Geraniol also gave a strong rose-like smell to treated fish samples. Therefore, the use of these essential oils as potential antimicrobial agents must consider the high concentrations needed which could result in pungent odors and discoloration. However, the need for high concentrations may be overcome by mixtures of antimicrobial compounds with synergistic action.

Using essential oil components for the inhibition of food pathogens may find a wider application in food processing. Bullerman et al. (1977) reported that cinnamic aldehyde and eugenol at levels used in candies and baked goods inhibited fungal growth. The antimicrobial effect of condiments added to fish sausage was believed to be attributed to essential oils (Subba et al., 1967). Dabbah et al. (1970) found orange oil extended the shelf life of milk. Liquid seasonings (sauces, dressings) containing emulsified essential oils and their components could also serve as antimicrobials against food pathogens.

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Edible Packaging Films Based on Fish Myofibrillar Proteins: Formulation and Functional Properties

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ABSTRACT

Biopackaging materials based on fish myofibrillar proteins have been developed. The effects of protein concentration, pH, temperature and storage time before casting on the apparent viscosity of the film forming solution (FFS) were evaluated using experimental design methodology. The first objective was to determine a feasible experimental range for film-forming. The pH and protein concentration had strong interactive effects on FFS viscosity. During FFS storage before casting, partial degradation of high molecular weight protein components led to decreased viscosity, allowing thin layer casting. In the experimental range for film-forming, none of the conditions affected film functional properties. Standard conditions were determined at: pH 3.0, 2.0g protein/100g FFS, 25°C and 6 hr storage. The functional properties of the standard biopackaging were slightly better than those that determined for known protein-based films, with tensile strength close to those of low density polyethylene films.

Key Words: biopackaging, edible film, myofibrillar proteins, mechanical properties, water vapor permeability

INTRODUCTION

FORMULATIONS for biomaterial-based packaging must include at least one component that will form a cohesive and continuous matrix (Gontard and Guilbert, 1994; Krochta et al., 1994; Cuq et al., 1995). Although not extensively studied, protein-based films have highly interesting properties. Animal proteins have been used historically to form edible barriers (e.g., sausage casings). Most research has focused on vegetable proteins. Many protein-based materials have been tested. These include, wheat gluten (Gontard et al., 1992), corn zein (Gennadios et al., 1993), soybean (Brandenburg et al., 1993), collagen (Cavallaro et al., 1994), ovalbumin (Guilbert, 1988), whey protein (McHugh and Krochta, 1994), and casein (McHugh et al., 1993).

The mechanical and barrier properties of protein-based films are generally better than those of polyosaccharide-based films. This is due to the fact that, contrary to polysaccharides which are homopolymers, proteins have a specific structure (based on 20 different monomers) which confers a wider range of potential functional properties, especially high intermolecular binding potential (Guilbert and Graille, 1994). They can form bonds at different positions, with different types and energies as a function of temperature, solvation conditions, pH, and additive characteristics (plasticizers, bonding agents, etc.). According to Kinsella (1976), Chou and Morr (1979), and Cheftel et al. (1985), experimental parameters such as protein concentration, pH, temperature, time, ionic strength, and presence of additives, modify the strengths of protein-protein and protein-water interactions and thus modify functional properties. The influence of these parameters has been evaluated by Lavelle and Foegeding (1993) on the gelation properties of myofibrillar proteins, and by Wu and Bates (1972) on film formation in a protein-lipid system. Moreover, protein-based raw materials can be chemically modified to improve functional properties (Osawa and Walsh, 1993).

High molecular weight proteins are generally insoluble or only weakly soluble in water and are thus very interesting film-forming molecules to form water resistant films. Among these, myofibrillar proteins have unusual film-forming properties (Okamoto, 1978). Myofibrillar proteins represent more than 50% of total muscle weight. They include contractile proteins (actin and myosin) and muscle contraction regulatory proteins (tropomyosin, troponin, actinin, etc.) and are characterized by their unusual functional properties (Cheftel et al., 1985). The functionalities of myofibrillar proteins at the molecular level have been described by Acton et al. (1983), Acton and Dick (1984), Regenstein (1984), and Whiting (1988).

Formation of protein-based films requires complete dissolution of proteins by adjusting the FFS pH or by suitable solvents. For instance, the most efficient pH for fish meat protein-based film formation has been established between pH 10.5 and 12 (Okamoto, 1978). Our first objective was to explore the potential film-forming properties of myofibrillar proteins and to define the experimental limits of important variables such as pH, protein concentration, temperature, and storage time. We also compared their functional properties with other protein-based films and synthetic films.

MATERIALS & METHODS

Preparation of fish mince

Washed fish mince was prepared from very fresh (1 day old) Atlantic sardines (*Sardina pilchardus*). Gutted and headed fish were passed through a meat bone separator (drum with 3 mm diameter perforations). The fish mince was washed twice using 1 part of mince for 4 parts of water (w/v). Each washing cycle was carried out for 15 min at 10°C, followed by straining in a rotary rinser before subsequent washing. The washed mince was passed through a refiner to remove final connective tissues and through a screw press to remove water and was chopped for 3 min in a cutter. The fish mince was then vacuum packed in polyethylene bags (500g) and kept at −23°C for a maximum of 1 mo. The samples were thawed for 24 hr at 4°C before the experiments.

Total protein of the washed fish mince was determined in triplicate by the Kjeldahl method (AOAC, 1980) using a conversion factor of 6.25. Lipid content was determined in triplicate by weighing after extraction in light petroleum for 3 hr (Osborne and Voogt, 1978). Moisture contents were determined in triplicate: 10g of washed fish mince were dried to constant weight in an oven at 104°C. The composition of the starting material was 76g (±1g) water, 23g protein (±1g), and 0.2g (±0.2g) lipid/100g fish mince.

Preparation of myofibrillar protein-based biopackagings

Films were prepared from a film-forming solution (FFS) based on fish mince in distilled water and acetic acid or ammonium hydroxide (Aldrich Chemie, Steinhem, Germany). Preliminary experiments were carried out to determine the critical values for each parameter: protein concentration (PC, with a minimal concentration of 0.5 g/100g FFS), FFS pH (adjusted with acetic acid or ammonium hydroxyde), temperature (T, °C) and storage time before casting (ST, hr). Glycerol at 35 g/100g dry matter (95%, Merck, Darmstadt, Germany) was added as plasticizer. This large amount of plasticizer was essential to obtain free standing flexible films.

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All components were mixed in a vacuum thermoregulated homogenizer (Stephan UM5, Marne la Vallée, France). FFS were stored for a few hours at the experimental temperature, before casting on a PVC plate using a thin-layer chromatography spreader to obtain films of 4 mg dry matter/cm². The thin layer was dried in a ventilated oven at 25°C and 50% RH for 15 hr. A transparent and easily handled film was thus formed.

Characterization of film-forming solutions

pH was determined with a ORION Research pH meter (Model EA920). The apparent viscosity of FFS was determined at experimental temperature, with a Brookfield viscosimeter (Model DV-II, Stoughton, USA). Measurements were carried out on FFS (200g in 250 ml beaker) using disc spindles. The probe rotation rate was 6.28 rad s^{-1}.

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on FFS according to Laemmli (1970) with some modifications for muscle system proteins (Roussel and Cheftel, 1990). The gradient concentration gels were 3.2% to 17.4% (w/v) acrylamide, 0.435M Tris-HCl (pH 8.7), 0.232% (w/v) SDS. The running buffer was 0.025M Tris-HCl (pH 8.7), 0.1% (w/v) SDS, 6M urea, 0.192M glycerol. Protein samples were denatured in 2.3% (v/v) 2-mercaptoethanol, 6.2% (w/v) SDS, 23.4% (w/v) sucrose, 1M Tris-HCl (pH 6.8), 7M urea, and incubated for 5 min at 90°C. Electrophoresis was conducted at 20°C using a vertical stand (Touzart & Matignon, model 6220 and AE-8400 Crosspower 1000). Gels were fixed and stained in a solution of 0.1% (w/v) Coomassie blue, 10% acetic acid, 45% methanol for 1 hr, and then destained overnight in a solution of 30% (w/v) methanol and 7.5% acetic acid. Molecular weight markers were from Sigma (St Louis, MO). The gels were scanned at 595 nm using a Hoeffer Scientific Instruments scanner (San Francisco, USA).

Characterization of myofibrillar protein-based biopackagings

Thickness was measured with a hand-held micrometer (Braive Instruments, Checy, France) with 7.5×10^{-3}m diameter faces, to the nearest 10^{-6}m. Thickness values represent means of five measurements (confidence interval = 4×10^{-6}m, at $p\leq0.05$).

Mechanical properties. Tensile strength and percentage elongation at break were determined using a Rheo TAXT2 Rheometer (Champlan, France), operated according to the ASTM standard method D 882-88 (ASTM, 1989). Protein-based films were equilibrated at 57% RH (using a saturated aqueous NaBr solution) and 25°C for 48 hr before testing. Samples (width = 10^{-2}m and length = 1.15×10^{-1}m) were coated with silicone grease to limit water vapor exchange with external atmosphere during measurements. Silicone coating had no effect on measurements. The rate of grip separation was 5×10^{-4}m.s^{-1}. Tensile strength (MPa) was calculated by dividing the peak load (N) by the cross-sectional area (mm²). Dividing the extension values by the initial distance between grips yielded percentage elongation at break. A total of 12 specimens was tested for each film type (confidence interval = 0.5 MPa for tensile strength and 2.1% for elongation at break, at $p\leq0.05$).

Water vapor permeability tests were conducted using a modified ASTM (1989) procedure presented by Gontard et al. (1992). The film was sealed in a glass permeation cell containing silica gel (0% RH). The cells were stored at 20°C in desiccators with distilled water. After steady-state conditions were reached, the cells were weighed at 24 hr intervals and water vapor permeability (WVP) of the film was calculated as follows:

$$\frac{\text{Water vapor permeability}}{(\text{mol}\cdot\text{m}\cdot\text{m}^{-2}\cdot\text{s}^{-1}\cdot\text{Pa}^{-1})} = \frac{w\cdot x}{A\cdot t\cdot(p_2-p_1)} \quad (1)$$

where w is the weight gain of the cup (g), t is the time of gain (s), x is the film thickness (m), A is the area of exposed film (m²), $p_2 - p_1$ is the vapor pressure differential across the film (Pa). There were at least five repetitions per experiment (confidence interval = 0.32 mol. m.m^{-2}.s^{-1}.Pa^{-1}, at $p\leq0.05$).

Statistical design

Response surface methodology (RSM) was used to determine the influence of experimental parameters on FFS properties. Theoretical and fundamental aspects of RSM applied in this field have been described by Gontard et al. (1992). The experimental design adopted was a modification of Box's central composite design for three variables each at five levels. The three independent variables were protein concentration (X_1), pH (X_2), and temperature (X_3). The dependent Y variable (response) under observation is FFS apparent viscosity. The actual values, chosen from preliminary experiments, and the corresponding coded values of the 3 independent variables are listed (Table 1). The complete design consisted of 18 points (realized randomly) which included four replications of the center point. Each value represents the mean of three replications. Data were analyzed to fit the following second order equation to the dependent Y variable:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (2)$$

where b_i are constant regression coefficients and X_1, X_2 and X_3 coded independent variables. The STAT GRAPHIC program (STSC's Software) was used for multiple regression analysis and evaluation of the goodness-of-the-fit. Response surfaces were drawn using the EXCEL 4.0 program (Microsoft Corporation) by using a constant value (middle level) for one of the three independent variables. The prediction power of the model was assessed by performing a separate experiment: the FFS was analyzed and experimental values were compared to model predictions.

Table 1—Responses of dependent variables to the film-forming conditions (means of three assays)

Design point	Independent variables[a]						Dependent variable
	X_1-Protein concentration (g/100g solution)		X_2-pH[b]		X_3-Temp (°C)		Y-Apparent viscosity (mPa · s)
	Coded	Actual	Coded	Actual	Coded	Actual	
1	−1	1.25	−1	2.75	−1	20	50
2	+1	2.75	−1	2.75	−1	20	48
3	−1	1.25	+1	3.25	−1	20	16700
4	+1	2.75	+1	3.25	−1	20	560
5	−1	1.25	−1	2.75	+1	40	320
6	+1	2.75	−1	2.75	+1	40	18
7	−1	1.25	+1	3.25	+1	40	19000
8	+1	2.75	+1	3.25	+1	40	5000
9	−2	0.50	0	3.00	0	30	12700
10	+2	3.50	0	3.00	0	30	182
11	0	2.00	−2	2.50	0	30	14
12	0	2.00	+2	3.50	0	30	27800
13	0	2.00	0	3.00	−2	10	133
14	0	2.00	0	3.00	+2	50	4300
15	0	2.00	0	3.00	0	30	57
16	0	2.00	0	3.00	0	30	70
17	0	2.00	0	3.00	0	30	58
18	0	2.00	0	3.00	0	30	56
VP	0	2.00	+1	3.25	0	30	60

[a] (X_1, X_2 and X_3 are independent variables, Y is a dependent variable, VP is a verification point).
[b] pH of the film-forming solution adjusted with acetic acid.

RESULTS & DISCUSSION

TO FORM FILM, myofibrillar proteins must not precipitate in FFS. The FFS must be characterized by a relatively low initial viscosity (initial apparent viscosity <700 mPa.s) in order to avoid retention of air bubbles. In addition, it must be characterized by a low viscosity at casting to be easily spread in thin layers.

Main influential process variables: preliminary experiments

The pH influence on FFS apparent viscosity was determined (Fig. 1). The apparent viscosity of FFS was greatly modified by pH. Apparent viscosity variations as a function of FFS pH could be attributed to redistribution of attractive and repulsive forces between proteins, due to variations in ionization state of the protein lateral chains (illustrated by variations of protein net charge as a function of pH, Fig. 1). This hypothesis was confirmed by the observed marked decrease in apparent viscosity, when the amino acid lateral chains were neutralized with NaCl at 10 g/100g FFS (Fig. 1). When NaCl was added, the proteins were salted-out and precipitated, leading to the decrease in viscosity.

The relative sensitivity of myofibrillar proteins to pH variations was due to the large proportion of ionizable amino acids:

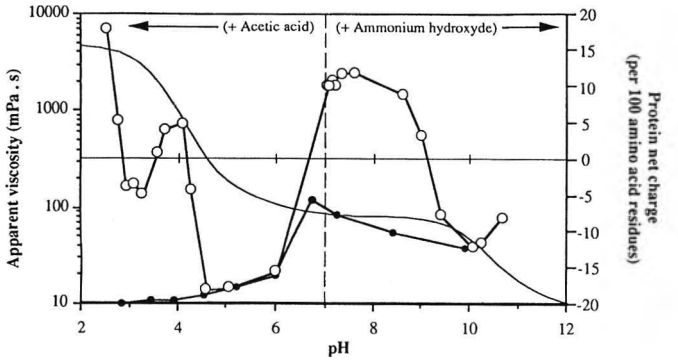

Fig. 1—pH influence on apparent viscosity of film-forming solution (with 2g of protein/100g film-forming solution at 25°C), adjusted with acetic acid or ammonium hydroxide, without (—○—), or with (—●—) addition of 10g NaCl/100g film-forming solution. (——) is the protein net charge.

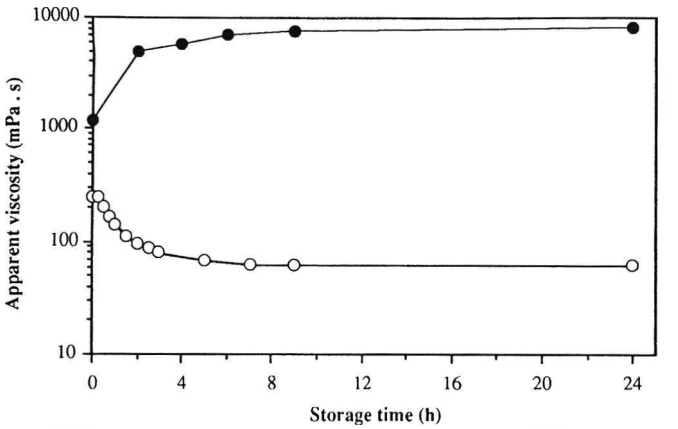

Fig. 2—Influence of storage time of film-forming solution on apparent viscosity, at pH 3.0, 2g of protein/100g film-forming solution at 20°C (—○—), or at pH 2.75, 2.75g of protein/100g film-forming solution at 20°C (—●—).

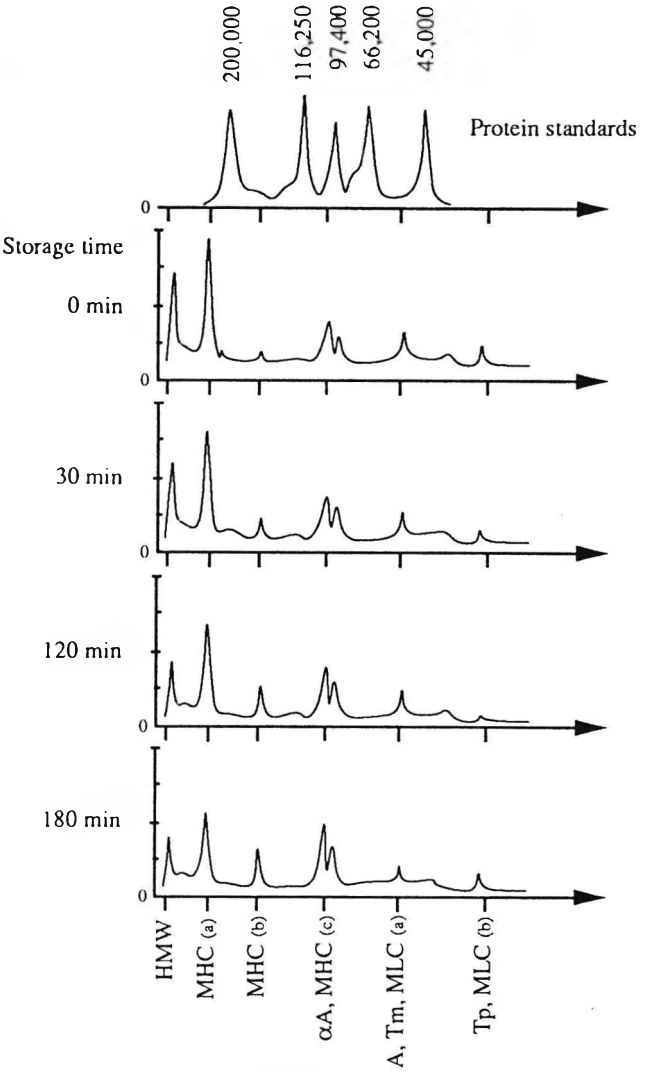

Fig. 3—Change of densitometer patterns of SDS-PAGE of film-forming solution for fish mince: HMW, high-molecular-weight fragment; MHC (a), myosin heavy chain (200 kd); MHC (b), myosin heavy chain fragment (140 kd); α-A, α-actinin (102 kd); MHC (c), myosin heavy chain fragment (70 kd); A, actin (45 kd); Tm, tropomyosin (35 kd); MLC (a), myosin light chain (25 kd); Tp, troponin (20–24 kd); MLC (b), myosin light chain (18 kd).

11.2g Asp, 17.8g Glu, 9.7g Lys, 1.9g His and 5.8g Arg/16g N (Orban et al., 1992). Formation of carboxylic groups by protonation of lateral chains of Asp and Glu during acidification induced a positive net charge as a result of constant lateral chain charges (NH_3^+) of basic amino acids. A similar behavior was observed during basification of FFS (Fig. 1), as a consequence of the negative net charge induced by deprotonation of basic amino acids and constant negative charges (COO^-).

The apparent viscosities of myofibrillar protein-based solutions were minimum between pH 4.5 and 6.5. In the isoelectric pH range (around pH_i 5.0), film formation was inhibited by myofibrillar protein precipitation. Similar behaviors were noted by Gennadios et al. (1993) for soy or wheat gluten protein-based film formation and by Okamoto (1978) with protein-based solutions, such as soybean, peanut, wheat, egg white, fish meat or keratin. The apparent viscosity of FFS increased substantially when pH decreased below 4.5 or increased to over 6.5 (Fig. 1). Orban ct al. (1992) showed that myofibrillar proteins were highly soluble, forming very viscous solutions at pH below 4.0 and at pH 9.0. At pH values out of the isoelectric range, proteins denature, unfold and solubilize, thus exposing interactive groups. Between pH 3.5 and pH 3.0, the apparent viscosity slightly decreased, then sharply increased at pH 2.2. These variations could be associated with molecular denaturations which are pH-dependent. A gel structure formed at pH <2.5. According to Hermansson et al. (1986) and Orban et al. (1992), controlled acidification of meat proteins is an effective method for producing gels at ambient temperature.

These experiments showed that the pH range for film-forming was limited by protein precipitation between pH 4.5 and 6.5 (or with NaCl addition) and by the high apparent viscosities (>700 mPa.s) between pH 6.5 and 9.0 and at pH <2.5. Films can be formed at pH between 2.5 and 4.0, and at pH >9.0 (apparent viscosities <700 mPa.s). Films obtained in basic conditions (pH >9.0) with a volatile base, such as ammonium hydroxide, were not used because of a persistent repulsive ammonia odor. Experiments were carried out with FFS acidified with acetic acid (less persistent odor), in the pH range which corresponds to the lower apparent viscosities in acidic conditions (between pH 2.5 and 4.0).

FFS storage time influence. Apparent viscosity variations of two different FFS (with high or low initial viscosity) were measured as a function of storage time. Apparent viscosity values were influenced by storage time (Fig. 2): solutions with a high initial viscosity (>700 mPa.s), (i.e., solutions at low pH and high protein concentration) showed an increase in apparent viscosity during storage. This could be associated with the formation of gel type structures, that hinders spreading of FFS in thin layers.

For FFS with low initial apparent viscosities (<700 mPa.s), a marked decrease in viscosity was observed during the first hours of storage (Fig. 2). This behavior allows transport and elimination of air bubbles. The apparent viscosity decrease could

Table 2—Regression coefficients and analysis of variance of the second order polynomial for the Y (apparent viscosity) response variable[a]

Coefficient		Y-Apparent viscosity	Error probability	
	b_0	58.5		
Linear	b_1	−6655	< 0.005	***
	b_2	11344	< 0.005	***
	b_3	1236	0.16	—
Interactions	b_{12}	−6732	< 0.005	***
	b_{13}	1809	0.10	*
	b_{23}	758	0.47	—
Quadratic	b_{11}	3009	0.01	***
	b_{22}	6742	< 0.005	***
	b_{33}	897	0.38	—
% Variability explained (R^2)		0.965		
F		2.46		
Probability of F		0.042		

[a] Model on which X_1 = protein concentration, X_2 = pH, and X_3 = temperature is: $Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$. * significant at 10% level. *** significant at 1% level.

Table 3—Mechanical properties of various films

Film[f]	Tensile strength (MPa)	Elongation (%)	Thickness ($\times 10^6$m)	Temp (°C)	RH (%)
Polyester[a]	178	85	—	—	—
Poly(vinylidene chloride)[a]	93.2	30	—	—	—
Cellulose acetate[a]	65.6	30	—	—	—
HDPE[a]	25.9	300	—	—	—
LDPE[a]	12.9	500	—	—	—
MC film[d]	56.1	18.5	—	25	50
Myofibrillar protein film	17.1	22.7	34	25	57
HPC film[d]	14.8	32.8	—	25	50
Whey protein isolate film[e]	13.9	30.8	—	23	50
Soy protein film-pH = 3[c]	1.9	35.6	88	25	50
Wheat gluten protein film-pH = 3[c]	0.9	260	88	25	50
Corn zein protein film[b]	0.4	—	81	26	50

[a] According to Briston (1988)
[b] According to Aydt et al. (1991)
[c] According to Gennadios et al. (1993)
[d] According to Park et al. (1993)
[e] According to McHugh and Krochta (1994)
[f] HPC is hydroxypropylcellulose, HPDE is high density polyethylene, LDPE is low density polyethylene, MC is methylcellulose.

be the result of degradation of high-molecular-weight proteins. Electrophoresis of FFS as a function of storage time before casting (at 0, 60, 120, and 180 min) showed modified electrophoresis scans (Fig. 3). As storage time increased, the myosin heavy chain (MHC) band intensity decreased and the density of the 140 kd (b) band increased. An increase in the 70 kd (c) band intensity, a decrease in densities of actin, a-actinin, troponin and tropomyosin bands, and a decrease in the intensity of very high-molecular-weight (e.g. connectin) bands were less pronounced changes. Similar results were reported by Saunders (1994) on myofibril proteins treated at acid pH for 20 hr, and these results could be explained by the action of acid-proteinases (mainly cathepsin) on myofibrillar proteins following acidification.

High-molecular-weight proteins are very sensitive to attack from cathepsins (Macfarlane et al., 1986; Saunders, 1994). Degradation of 200 kd bands could be attributed to MHC breakdown, the only component present in sufficient amount to give rise to bands of this intensity. According to Saunders (1994), who studied the influence of pH on MHC degradation at 5°C, pH 3.0 is optimum for loss of MHC, and corresponds to optimum activity of cathepsins on myofibrillar proteins (Matsumoto et al., 1983). The presence of proteolytic enzymes in fractionated meat systems has not been reported in detail, but Saunders (1994) explained that these enzymes could become strongly associated with the myofibrillar fraction and were not removed by extensive washing, during the preparation of minced meat with aqueous extraction (Young et al., 1992).

Table 4—Water vapor permeability of various films

Film[j]	Water vapor permeability[k]	Temp (°C)	Thickness ($\times 10^6$ m)	RH % conditions
Starch, cellulose acetate film[a]	142	38	1190	100-30
Sodium caseinate film[b]	24.7	25	—	100-00
Soy protein film-pH = 3[c]	23.0	25	83	100-50
Corn zein film[d]	6.45	21	200	85-00
HPMC film[e]	5.96	27	19	85-00
MC film[f]	5.23	30	75	11-00
Wheat gluten film[g]	5.08	30	50	100-00
Myofibrillar protein film	3.91	25	60	100-00
HPC film[f]	2.89	30	75	11-00
LDPE film[h]	0.0482	38	25	95-00
Wheat gluten-beeswax-bilayer[g]	0.0230	30	90	100-00
HDPE film[h]	0.0122	38	25	97-00
Beeswax film[i]	0.0122	25	120	87-00

[a] According to Allen et al. (1963)
[b] According to Avena-Bustillos and Krochta (1993)
[c] According to Gennadios et al. (1993)
[d] According to Park and Chinnan (1990)
[e] According to Hagenmaier and Shaw (1990)
[f] According to Park et al. (1993)
[g] According to Gontard et al. (1994)
[h] According to Myers et al. (1961)
[i] According to Landman et al. (1960)
[j] HPC is hydroxypropylcellulose, HPDE is high density polyethylene, HPMC is hydroxypropyl methylcellulose, LDPE is low density polyethylene, MC is methylcellulose.
[k] 10^{-12} mol · m · m^2 · s^{-1} · Pa^{-1}

These experiments revealed the need for a FFS "maturation" step for air bubble elimination, before spreading the solution in thin layers, when a degassing step appeared to be in effective. To form protein-based films, FFS with a low initial viscosity (<700 mPa.s) must be stored at ambient temperature for at least 6 hr before casting. Determination of the functional properties of some films formed at various storage times (between 6 and 24 hr) showed that additional storage time was not necessary. Tensile strength, percent elongation, and water barrier properties remained constant (close to those presented in Tables 3 and 4). In further experiments, all FFS were stored for 6 hr before spreading in thin layers.

Influence of pH, protein concentration, and temperature

Response surface methodology was used to determine the influence of pH, protein concentration and temperature on FFS apparent viscosity. The experimental data (Table 1) were analyzed to fit Eq. (2). Table 2 summarizes the results of the analysis of variance for dependent variables with corresponding coefficients of multiple determination (R^2).

The model designed for apparent viscosity seemed suitable, with satisfactory R^2 value (0.965) and significant F value ($p=0.05$). Careful assessment of residuals indicated that a higher order model would be useless. The estimated partial regression coefficients and the results of significance tests on the coefficients are given in Table 2. The relationship between independent and dependent variables is represented by a 3-dimensional response surface (Fig. 4) generated by the regression equations formulated from the mathematical analysis. Adequacy of the models was tested by performing a separate experiment under the process conditions listed (Table 1). Results showed close agreement of the experimental and simulated values.

Judging from the significant regression coefficients (Table 2), temperature did not appear to be the prime factor affecting FFS apparent viscosity; the effect of temperature was only observed in association with protein concentration. However, note that the influence of temperature could be masked by the other two parameters (pH and protein concentration) which were more influential. Moreover, according to Lanier et al. (1982), the effect of temperature on myofibrillar protein structure was only noted for temperatures over 40°C and it became very important at 50°C. FFS thermal gelation was observed during storage at 50°C, preventing spreading in thin layers. These results were in full agree-

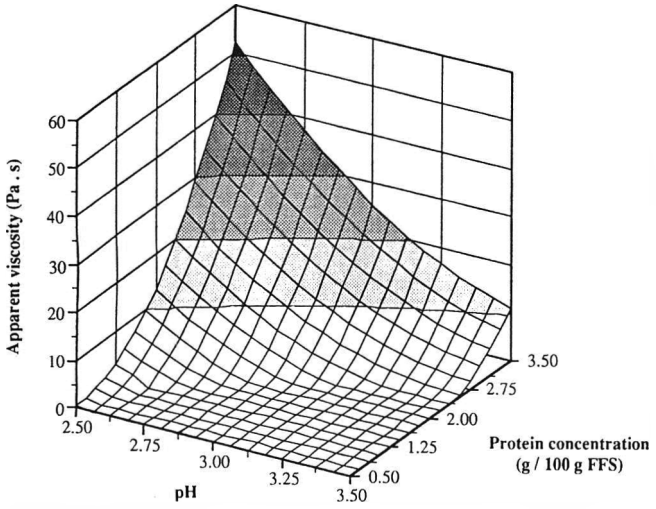

Fig. 4—Response surface for the effect of pH and protein concentration on apparent viscosity of film-forming solution at 30°C.

ment with data on thermal denaturation of myofibrillar proteins for relatively high temperatures (Foegeding, 1988; Beas et al., 1990). This indicates that experimental temperatures to form myofibrillar protein-based biopackagings must be <50°C.

Determination of the functional properties of some films formed at various temperatures (between 10 and 40°C) showed that this parameter was not influential. Tensile strength, percent elongation, and water barrier properties remained constant values (close to those presented in Tables 3 and 4). For further experiments, the temperature was fixed at 25°C (ambient conditions).

The main factors influencing apparent viscosity were pH and protein concentration, with their linear and quadratic coefficients and the interaction coefficient (Table 2). The shape of the response surface (Fig. 4) indicates a strong interaction between these two variables and shows that the lowest apparent viscosity value could be expected with low protein concentrations and relatively high pH. On the response surface, the experimental conditions involving high protein concentrations and low pH, led to formation of very viscous FFS with gel structure formation at critical values. These conditions could not be applied for film formation. Conditions leading to low viscosity FFS, that were to be used to form films, involved pH values between 2.75 and 3.5, and PC values between 0.5 and 2.5 g/100g FFS. The experimental fabrication range for myofibrillar protein-based biopackagings appears to be quite limited in contrast to wheat gluten- or soybean protein-based films (Gontard et al., 1992; Gennadios et al., 1993).

Determination of functional properties showed that these parameters were not influential, and that tensile strength, percent elongation and water barrier properties remained constant values (close to those presented in Tables 3 and 4). For further experiments, pH was fixed at 3.0 and protein concentration at 2.0 g/100g FFS. None of the experimental conditions noted above affected final film properties, they only affected the ability to form films. All films exhibited similar functional properties no matter how they were formed. Standard experimental conditions for forming myofibrillar protein-based biopackagings from a film-forming solution were so determined at pH 3.0, 2.0g of protein/100g FFS, 25°C and 6 hr storage time. The resulting films had interesting functional properties.

Functional properties of standard myofibrillar protein-based biopackagings

The mechanical properties (tensile strength and elongation at break) of myofibrillar protein-based biopackagings and of various films were compared (Table 3). Myofibrillar protein-based biopackagings have mechanical properties similar to those of various biomaterial-based films. The mechanical properties of myofibrillar protein-based biopackagings are close to those of polysaccharide-based films (such as elongation at break for methylcellulose-based films and tensile strength for hydroxy propylcellulose-based films). Tensile strength was slightly above (films were more mechanically resistant) and elongation was slightly below (films were less deformable) values determined with already knowr. protein-based films (Table 3 for whey, soy, wheat and corn protein-based films). Myofibrillar protein-based biopackagings have substantially lower tensile strength (17.1 MPa) than polymeric materials such as poly(vinylidene chloride), cellulose acetate, and polyester (respectively 93.2, 65.6 and 178 MPa), but are relatively close to values of polyethylene synthetic films (12.9 and 25.9 MPa, respectively for low density and high density polyethylene). Myofibrillar protein-based biopackagings have a lower percentage elongation (22.7%) than poly(vinylidene chloride), cellulose acetate, polyester and polyethylene films (Table 3).

Water vapor permeabilities of myofibrillar protein-based biopackagings and of various films are presented (Table 4). Myofibrillar protein-based biopackagings were characterized by relatively poor water vapor barrier properties (WVP = 3.5×10^{-12} mol.m.m^{-2}.s^{-1}.Pa^{-1}), like all hydrocolloid-based films. The permeability values were slightly lower than values determined for protein-based films (Table 4 for wheat gluten, soy, caseinate and corn zein protein-based films). Resistance of protein-based films to water vapor transmission is limited due to the inherent hydrophilicity of proteins. Transmission of water vapor through protein-based films is also facilitated by the presence of glycerol, a hydrophilic plasticizer, which favors adsorption of water molecules. Water vapor permeability constant values determined in this study for myofibrillar protein-based biopackagings were higher by 10^2 as compared to those of typical polymeric packaging materials (Table 4), (WVP = 4.8×10^{-10} and 1.2×10^{-10} mol.m.m^{-2}.s^{-1}.Pa^{-1}, respectively for low density and high density polyethylene films).

CONCLUSION

MYOFIBRILLAR PROTEIN-BASED BIOPACKAGINGS could be cast in thin layers from a film-forming solution. These films are characterized by interesting functional properties which are however lower than standard synthetic films. The feasibility of using myofibrillar protein as a film forming-agent appeared promising considering the lack of appearance problems. The overall film properties, and possible improvement of those properties through optimization of other parameters (such as amount and type of plasticizers) are feasible.

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This research was supported by a grant from the Ministère de l'Enseignement Supérieur et de la Recherche (Contrat Aliment 2002, no. 92.G.0672, 11/18/92).

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Ms received 3/20/95; revised 6/20/95; accepted 7/8/95.

Florida Agricultural Experiment Station Journal Series No. R-04439.

1995/VOLUME 60

JOURNAL OF FOOD SCIENCE

(formerly Food Research)

AN OFFICIAL PUBLICATION OF THE

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ELECTRONIC PROCESSING-Authors are requested to furnish an IBM compatible disk (3-1/2 in. or 5-1/4 in., Word Perfect software and DOS or ASCII text) of their accepted manuscript to further facilitate processing. A "Guide to Submission of JFS Manuscripts on Disk" was published in J. Food Sci. 60(5): iv-v (1995). Send the disk and copy of the revised manuscript to Dr. R.E. Berry, Scientific Editor. Although not mandatory, compliance with this request will expedite publication.

Rev. 11, 11/95

Guidelines for the Preparation and Review of Research Papers on Fruit and Vegetable Products

by the IFT Fruit & Vegetable Products Division

☐The Fruit & Vegetable Products Division of the Institute of Food Technologists has prepared the following guidelines for use by authors in preparing research papers on fruit and vegetable products for publication in *Journal of Food Science*, as well as for use by reviewers when evaluating the suitability of such manuscripts for publication. These guidelines—prepared by Sara E. Spayd and approved by the Fruit & Vegetable Products Division executive committee—are intended to supplement the "Style Guide for Research Papers" published in *Journal of Food Science* 60: 1397-1400 (1995).

Guidelines

Manuscripts resulting from original research on horticultural commodities that are destined for fresh or processed products for human consumption are to be considered for acceptance and publication in the *Journal of Food Science.* Relationships of the study to use the commodity as human food must be clearly established. This may be included in the introductory remarks, discussion comments, and/or referenced previous reports. Research publications concerning fresh fruits and vegetables may include, but are not limited to, pre- and post-harvest agronomic, environmental, handling, or storage variables that directly affect the ultimate consumer quality and/or shelf life of the fruit or vegetable. Research manuscripts on processing technologies may also include pre- and post-harvest variables that directly influence the consumer quality or shelf life of processed fruits and vegetables. Increased interest in the relationships among agronomic, postharvest, and processing variables and the nutrient, safety, shelf-life, and health-promoting qualities of fruits and vegetables justifies their editorial consideration for publication in *Journal of Food Science.*

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(Required by 39 U.S.C. 3685)

1. Publication Title	2. Publication No.	3. Filing Date
JOURNAL OF FOOD SCIENCE	0 0 8 7 - 2 2 8 0	9/7/95
4. Issue Frequency	**5. No. of Issues Published Annually**	**6. Annual Subscription Price**
BI-MONTHLY (EVERY OTHER MONTH)	6	$100.00

7. Complete Mailing Address of Known Office of Publication *(Street, City, County, State, and ZIP+4) (Not Printer)*

INSTITUTE OF FOOD TECHNOLOGISTS
221 N. LASALLE STREET, SUITE 300
CHICAGO, COOK COUNTY, IL 60601

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JOURNAL OF FOOD SCIENCE	JULY/AUGUST, 1995	
15. Extent and Nature of Circulation	Average No. Copies Each Issue During Preceding 12 Months	Actual No. Copies of Single Issue Published Nearest to Filing Date
a. Total No. Copies *(Net Press Run)*	11,091	11,080
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