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CHEMICAL DETERIORATION OF FROZEN BOVINE MUSCLE AT −4°C. A. AWAD, W. D. POWRIE & O. FENNEMA. *J. Food Sci.* **33**, 227–235 (1968)—During an 8-week storage period of bovine muscle at −4°C, the total extractable protein content dropped from 91 to 51%. This decline, accompanied by a decrease in water-binding capacity of muscle, was primarily caused by the insolubilization of both sarcoplasmic proteins and actomyosin. The increase in the free fatty acid content from about 1.6 to 9.1% over the 8-week period may be attributed to phospholipid hydrolysis. The TBA number rose progressively throughout the entire storage period while the peroxide value increased to a maximum in 2 weeks of storage and then decreased to a constant value of 7.

TEMPERATURE ACCLIMATION AND ITS EFFECTS ON PORCINE MUSCLE PROPERTIES IN TWO HUMIDITY ENVIRONMENTS. JEAN M. HOWE, N. W. THOMAS, P. B. ADDIS & M. D. JUDGE. *J. Food Sci.* **33**, 235–238 (1968)—Exposure to ambient temperatures of 32 and 21°C for alternating 3-day periods caused rapid post-mortem glycolysis, high percent light reflectance, and increased light to dark fiber ratios in the longissimus dorsi muscle as compared to constant (27°C) temperature, but only in moderate (38–42% relative) humidity environments. No significant differences were found in lactic dehydrogenase or succinic dehydrogenase enzyme activities of longissimus dorsi or gluteus medius muscles.

DEVELOPMENT OF AN ISOTONIC-ISOMETRIC RIGOROMETER. G. R. SCHMIDT, R. G. CASSENS & E. J. BRISKEY. *J. Food Sci.* **33**, 239–241 (1968)—The rigorometer is an enclosed, temperature controlled, environmental chamber designed specifically for studies on strips of parallel muscle fibers. The chamber will accommodate six muscle strips of varying sizes and has a wide adjustment for degree of sensitivity. Small cylinders have also been specially designed to facilitate separate controls of aqueous and gaseous environments surrounding each muscle strip. The details of the design and application of this rigorometer are discussed.

RATE OF DETERIORATION OF FREEZE-DRIED SALMON AS A FUNCTION OF RELATIVE HUMIDITY. F. MARTINEZ & T. P. LABUZA. *J. Food Sci.* **33**, 241–247 (1968)—The rates of several deterorative reactions, including lipid oxidation, astacene pigment loss, carbon dioxide production, and production of non-enzymatic browning pigments, were studied in freeze-dried salmon at 37°C and at several relative humidities. Both the rate of the initial peroxide monomolecular decomposition and the peroxide value decreased as the water content was increased. Astacene pigment loss was reduced significantly by the higher moisture contents; non-enzymatic browning was increased. The significance of the reactivity of water at low moisture contents was demonstrated by its effect on the various reactions.

CELL DISRUPTION IN BROILER BREAST MUSCLE RELATED TO FREEZING TIME. J. C. CRIGLER & L. E. DAWSON. *J. Food Sci.* **33**, 248–250 (1968)—The degree of cell disruption was estimated after measuring the amount of drip released and by total solids, nitrogen and deoxyribonucleic acid (DNA) concentration of the drip. In general, increased freezing time resulted in greater cell disruption; however, several exceptions were noted. Cell disruption was relatively severe for tissues frozen in 18 to 35, 87, and 252 min, and relatively low for tissues frozen in times of 1 to 18 min, 132 to 225 min, and longer than 1,044 min. All frozen and thawed muscles had higher contents of total solids, nitrogen and DNA than unfrozen controls.

PEANUT ALCOHOL DEHYDROGENASE. 1. Isolation and Purification. H. E. PATTEE & H. E. SWAISGOOD. *J. Food Sci.* **33**, 250–253 (1968)—Alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase EC 1.1.1.1) has been isolated and purified from peanut kernels. The resulting preparations exhibited a high degree of purity as shown by the criteria of ultracentrifugation and free boundary and zonal electrophoresis. The simultaneous purification of zinc and enzymic activity indicates that peanut alcohol dehydrogenase is a zinc metalloenzyme.

THE CHEMICAL NATURE AND PRECURSORS OF CLARIFIED APPLE JUICE SEDIMENT. G. JOHNSON, B. J. DONNELLY & K. JOHNSON. *J. Food Sci.* **33**, 254–257 (1968)—Chemical analysis of sediment formed in clarified apple juice upon storage showed that it was composed of a polymeric phenolic fraction with which a varying amount of protein was associated. The variable nitrogen, mineral and ash contents of different sediments, and the variable amino acid composition of the protein fraction in conjunction with the behavior of the sediments on Sephadex gel columns indicated the heterogeneous nature of this material. Polyamide thin-layer chromatography and colorimetric analyses showed that the leuco-anthocyanidins and catechins are the main precursors of the polymeric phenolic component while chlorogenic acid appears to play an insignificant role in sediment formation.

GAMMA RADIATION EFFECTS ON THE PECTIC SUBSTANCES IN CITRUS FRUITS. A. H. ROUSE & R. A. DENNISON. *J. Food Sci.* **33**, 258–261 (1968)—Exposure of Valencia oranges and Duncan grapefruit to radiation doses of 150 and 300 krad increased the water- and oxalate-soluble pectins and decreased the protopection in component parts of the fruits. Quantities of water-soluble pectin and protopectin in the peel and membrane were affected by increased dosages in both fruits. Pectins were degraded in all of the components of the treated fruits and the methoxyl contents of the pectins from the peel of oranges and grapefruit decreased. Pectinesterase (PE) activity in the peel of the citrus fruits decreased with increased dosage, while the PE in the membrane increased with dosage.

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ESTER CONTENT AND JELLY pH INFLUENCES ON THE GRADE OF PECTINS. C. J. B. SMIT & E. F. BRYANT. *J. Food Sci.* **33**, 262–264 (1968)—A series of pectin samples, demethylated under mild acid conditions, showed no change in molecular weight or galacturonic acid content during demethylation. Setting times increased until an ester content of 50% was reached and at lower ester levels it decreased again. The maximum jelly grade obtained with a particular sample decreased and the pH at which this maximum grade could still be obtained increased as the ester content increased above *ca.* 45%. Below this ester level, the maximum grade as well as the maximum pH at which this grade could be obtained decreased with ester content.

QUANTITATIVE METHODS FOR ANTHOCYANINS. 3. Purification of Cranberry Anthocyanins. T. FULEKI & F. J. FRANCIS. *J. Food Sci.* **33**, 266–274 (1968)—Lead acetate precipitation, polyamide and ion exchange resin column chromatography were evaluated from a quantitative viewpoint for the purification of anthocyanins in cranberry juice cocktail. The evaluation of the various purification methods was based, in order of their importance, on the recovery of individual and total anthocyanins and on the concentrating power. Amberlite CG-50 ion exchange resin was the best, but basic lead acetate was also satisfactory. Polyamide did not concentrate the anthocyanins and the use of neutral lead acetate resulted in poor recoveries.

FLAVONOID COMPOUNDS IN THE STRAWBERRY FRUIT. HENRY CO & P. MARKAKIS. *J. Food Sci.* **33**, 281–283 (1968)—Flavonoid compounds of the strawberry fruit were studied by paper chromatography, spectrophotometry, and color reactions. Catechin, quercetin-3-glucoside, kaempferol-3-glucoside, and leucocyanidins of varying degrees of polymerization were found besides the two anthocyanins, pelargonidin-3-glucoside and cyanidin-3-glucoside.

STUDIES ON THE AROMA OF INTACT HAMLIN ORANGES. J. A. ATTAWAY & M. F. OBERBACHER. *J. Food Sci.* **33**, 287–289 (1968)—Volatile aromatic compounds emitted from oranges on storage and less volatile aroma compounds from the cuticle of the fruit were isolated and analyzed. The volatile aroma of the stored oranges was contributed chiefly by ethyl esters, while sesquiterpene hydrocarbons appeared to be responsible for the persistent odor from the cuticle.

VOLATILE COMPOUNDS FROM HEATED GLUCOSE. R. H. WALTER & I. S. FAGERSON. *J. Food Sci.* **33**, 294–297 (1968)—Volatiles from anhydrous glucose heated in air at 250°C for 30 min were collected in a trap maintained at the temperature of solid carbon dioxide. A concentrated ether extract of the distillate was shown by gas chromatography to contain at least 100 compounds. Among those identified and heretofore unreported from heated glucose were the gamma lactone of 4-hydroxy-2-pentenoic acid, 1-(2'-furyl)-propane-1,2-dione (acetylfuroyl), 3-methylcyclopentane-1,2-dione, phenol and methylfuroic acid.

OLFACTORY THRESHOLD IN RELATION TO AGE, SEX OR SMOKING. D. VENSTROM & J. E. AMOORE. *J. Food Sci.* **33**, 264–265 (1968)—The individual olfactory thresholds of 97 persons towards 18 odorants have been analyzed statistically. There is a significant logarithmic deterioration with age, the average loss of sensitivity being 50% in 22 years. Any influence of sex or smoking is negligible.

AUTO-OXIDATION OF EXTRACTABLE COLOR PIGMENTS IN CHILI PEPPER WITH SPECIAL REFERENCE TO ETHOXYQUIN TREATMENT. S. L. CHEN & F. GUTMANIS. *J. Food Sci.* **33**, 274–280 (1968)—Deterioration of extractable color pigments of dehydrated, ground chili peppers during storage was shown to be an auto-oxidative precess having the kinetics of a second order reaction. Consequently, the reaction rate constant, k_2, was used to evaluate the effect of a number of variables, such as moisture content, storage atmosphere and ethoxyquin treatment. It also provided a means for comparing the relative color stability of different pepper varieties.

VOLATILE COMPONENTS OF PINEAPPLE. R. K. CREVELING, R. M. SILVERSTEIN & W. G. JENNINGS. *J. Food Sci.* **33**, 284-287 (1968)—By gas chromatographic retentions, infrared spectroscopy and where applicable, mass and nmr spectroscopy, several additional compounds have been identified as components of pineapple essence. These are acetoxyacetone, dimethyl malonate, *trans*-tetrahydro-α, α,5-trimethyl-5-vinyl furfuryl alcohol, methyl *cis*-(4?)-octenoate, γ-butyrolactone, methyl β-hydroxybutyrate, methyl and ethyl β-hydroxyhexanoate, methyl and ethyl β-acetoxyhexonate, γ-octalactone, and δ-octalactone. Possible biogenic pathways to some of these compounds are discussed.

VOLATILE FLAVOR COMPONENTS FROM GREEN PEAS *(PISUM SATIVUM)*. 1. Alcohols in Unblanched Frozen Peas. K. E. MURRAY, J. SHIPTON, F. B. WHITFIELD, B. H. KENNETT & G. STANLEY. *J. Food Sci.* **33**, 290–294 (1968)—Positively identified were: methanol, ethanol, propan-1-ol, butan-1-ol, butan-2-ol, 2-methylpropan-1-ol, pentan-1-ol, pentan-3-ol, 2-methylbutan-1-ol, 3-methylbutan-1-ol, hexan-1-ol, heptan-1-ol, octan-1-ol, pen-1-en-3-ol, *cis* and *trans* pent-2-en-1-ol, *cis* and *trans* hex-3-en-1-ol, *trans* hept-2-en-1-ol, *trans* oct-2-en-1-ol, oct-1-en-3-ol and *cis* non-3-en-1-ol. Tentative identification was made of *trans* hex-2-en-1-ol, *cis* and *trans* hept-3-en-1-ol and nonan-1-ol.

COMPARATIVE DISTRIBUTION OF VOLATILE ALIPHATIC DISULFIDES DERIVED FROM FRESH AND DEHYDRATED ONIONS. RICHARD A. BERNHARD. *J. Food Sci.* **33**, 298–304 (1968)—Gas chromatographic determination of the volatiles from fresh "Sunspice" onions revealed that the principal disulfide present is di-*n*-propyl, followed in descending order of concentration by *n*-propyl allyl, methyl-*n*-propyl, methyl allyl, dimethyl, and diallyl. In dehydrated onions this order is markedly altered. Methyl-*n*-propyl is the principal disulfide, followed by dimethyl, methyl allyl, di-*n*-propyl, *n*-propyl allyl, and diallyl. Quantitative estimates of the disulfide content of 53 lots of fresh onions were made and compared with analyses of dehydrated onions from comparable lot numbers. Loss of total volatiles averaged 98%, while loss of disulfides was greater than 89%. The relationship of disulfides to onion flavor and a pungency ranking system based on these analyses are discussed.

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SPECIFICITY IN THERMAL HYDROLYSIS OF TRIGLYCERIDES. C. BUZIASSY & W. W. NAWAR. *J. Food Sci.* **33**, 304–306 (1968)—Triglyceride mixtures were heated in the presence of water under controlled conditions and the released fatty acids quantitatively analyzed by gas chromatography. Experiments with both a mixture of monoacyl-triglycerides and glycerides with equimolar amounts of randomly distributed fatty acids showed a preference for the hydrolysis of the shorter chain and the unsaturated fatty acids. The C_4, C_8, C_{12}, C_{16} and $C_{18:1}$ fatty acids were used in the above mixtures. Trilaurin, in which the fatty acid in the 2-position is labelled with C^{14}, was synthesized. When the free acids released by heat were analyzed by a combination gas chromatograph-flow counter detector system, no evidence for a positional specificity was apparent.

ACID-SOLUBLE NUCLEOTIDES OF KING CRAB MUSCLE. R. W. PORTER. *J. Food Sci.* **33**, 311–314 (1968)—The nucleotides in rested king crab *(Paralithodes camschatica)* muscle were identified as NAD, AMP, GMP, IMP, ADP, UDPAG, UDPG, ATP, GTP, and UTP. Two additional minor nucleotide fractions are tentatively thought to contain NAD; a third remains unidentified. Nucleotide profiles of three different leg sections were very similar, with ATP predominant. The average total nucleotide content of the three sections was 3.47 $\mu M/g$. Muscle from severely exhausted crabs held frozen overnight contained 0.35 $\mu M/g$ of IMP. Since king crabs are processed fresh and IMP does not accumulate to a very high level, IMP probably is not important in king crab flavor.

FREEZE-DRYING CAKE BATTER FOR MICROSCOPIC STUDY. P. H. POHL, A. C. MACKEY & B. L. CORNELIA. *J. Food Sci.* **33**, 318–320 (1968)—A method is reported for preparing cake batter for microscopic study through freeze-drying, followed by fixation and staining of fat with osmium tetroxide, infiltration with paraffin, and sectioning of the freeze-dried batter. Microscopic examination reveals that a cake batter is an emulsion of fat in an aerated aqueous phase. The fat particles are irregularly "globular" shaped droplets dispersed throughout the aqueous starch-protein system. In like manner, the air bubbles are dispersed in the batter. They are not incorporated in the fat particles, but are distributed throughout the aqueous phase.

HISTOLOGICAL DEVELOPMENT OF THE GREEN BEAN POD AS RELATED TO CULINARY TEXTURE. 2. Structure and Composition at Edible Maturity. R. M. REEVE & M. S. BROWN. *J Food. Sci.* **33**, 326–331 (1968)—Inner parenchyma and fiber sheath tissues are histologically younger than outer parenchyma, skin and fibrous bundle tissues of the pod at early edible maturities. Growing conditions, variety, and temperature of postharvest storage influence cell wall composition and texture. Cellulosic changes during drying increases toughness. Substomatal spaces, intercellular spaces, and structural differentiation of epidermis and hypodermis contribute to skin sloughing, which is further influenced by pectic composition.

BIOLOGICAL EVALUATION OF PROTEIN QUALITY OF RADIATION-PASTEURIZED HADDOCK, FLOUNDER AND CRAB. E. F. REBER, M. H. BERT, E. M. RUST & E. KUO. *J. Food Sci.* **33**, 335–337 (1968)—The effects of radiation sub-sterilization or cooking on the protein quality of haddock, crab, and flounder were evaluated by determining the protein efficiency ratio (PER). The PER value of haddock radiated at either level was statistically equal to that of non-radiated haddock. The PER value of crab radiated at either level was statistically equal to non-radiated crab in one replication and significantly higher than non-radiated crab in another replication. The PER value of flounder radiated at the low level was significantly less than that of non-radiated flounder, whereas the PER value of flounder radiated at the high level was statistically equal to that of non-radiated flounder.

ALDITOL DETERMINATION IN THE PRESENCE OF SACCHARIDES. O. SAMUELSON & H. STRÖMBERG. *J. Food Sci.* **33**, 307–310 (1968)—Alditols and sugars were extracted with water from sweets, and after dilution with ethanol the solutions were subjected to partition chromatography on ion-exchange resins. The alditols and monosaccharides were determined automatically using the periodate and orcinol methods.

QUANTITATIVE DETERMINATION OF BOUND WATER BY NMR. R. TOLEDO, M. P. STEINBERG & A. I. NELSON. *J. Food Sci.* **33**, 315–316 (1968)—The bound water content, defined as that which remained liquid at 0°F (−18°C), was 0.29 ± 0.01 g water per g dry solid in case of a wheat flour dough. This value was independent of total moisture content for doughs of the same flour with moisture contents greater than 24.6%. NMR signals indicated that most of this water remained liquid at −58°F (−50°C). This method gives a direct reading of bound water and is nondestructive of sample.

HISTOLOGICAL DEVELOPMENT OF THE GREEN BEAN POD AS RELATED TO CULINARY TEXTURE. 1. Early Stages of Pod Development. R. M. REEVE & M. S. BROWN. *J. Food Sci.* **33**, 321–326 (1968)—Vascular bundles and associated fibers differentiate in dorsal and ventral suture areas of the pods. Cell divisions are frequent in young inner parenchyma even after divisions have ceased in the outer parenchyma. All divisions cease as pods approach edible maturity and further growth is by cell enlargement. Compositional differences accompanying differences in rates of tissue maturation relate to texture problems.

THE NUTRITIVE VALUE OF HUMANIZED MILK FOOD BASED ON BUFFALO MILK AND FORTIFIED WITH DL-METHIONINE. V. A. DANIEL, B. L. M. DESAI, S. VENKAT RAO, M. SWAMINATHAN & H. A. B. PARPIA. *J. Food Sci.* **33**, 331–334 (1968)—The results indicate that humanized milk food from buffalo milk containing about 12% for protein and 20% fat and fortified with DL-methionine will be suitable for feeding infants in place of full cream milk powder in developing countries where milk is in short supply. Adoption of the above formula for infant milk food manufactured in the country will help to *double* the output of infant food from the same quantity of buffalo milk without appreciable increase in cost.

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A. AWAD,[a] W. D. POWRIE and O. FENNEMA
Department of Food Science and Industries
University of Wisconsin, Madison 53706

Chemical Deterioration of Frozen Bovine Muscle at -4° C

SUMMARY—During an 8-week storage period of bovine muscle at −4°C, the total extractable protein content dropped from 91 to 51%. This decline, accompanied by a decrease in water-binding capacity of muscle, was primarily caused by the insolubilization of both sarcoplasmic proteins and actomyosin. The increase in the free fatty acid content from about 1.6 to 9.1% over the 8-week period may be attributed to phospholipid hydrolysis. The TBA number rose progressively throughout the entire storage period while the peroxide value increased to a maximum in 2 weeks of storage and then decreased to a constant value of 7.

INTRODUCTION

DURING FREEZING and frozen storage of muscle, microstructural and chemical alterations are evident (Connell, 1964; Fennema *et al.*, 1964; Khan, 1967; Love, 1966). In general, the consequences of these changes may be exudation of fluid (drip) from thawed muscle, loss of muscle tenderness and development of rancid odor.

With regard to frozen bovine muscle, a major concern of researchers has been the relationship of freezing conditions to amount of drip. According to Callow (1952), Cook *et al.* (1926) and Hiner *et al.* (1945), the amount of drip from bovine muscle was reduced when the freezing rate was increased. Moran (1932) reported that when small pieces of bovine muscle were frozen in liquid nitrogen, only a small amount of drip was collected. Ramsbottom *et al.* (1939) emphasized that the freezing rate-drip relationship existed only when the volume of beef was small in comparison with the cut surface area.

The data of Moran *et al.* (1932) indicated that the amount of drip from frozen beef increased as the storage temperature was elevated. On the other hand, Ramsbottom *et al.* (1941) found no relationship between frozen storage temperature and drip from beef. According to Ramsbottom *et al.* (1940), the amount of drip from previously-frozen beef steaks decreased as the prefreezing aging period was extended up to 35 days. Sair *et al.* (1938) reached a similar conclusion. Empey (1933), Sair *et al.* (1938) and Ramsbottom *et al.* (1940) indicated that little or no drip was evident when the pH of beef muscle was between 6.2 and 6.4. However, as the pH decreased, the volume of drip gradually increased to a maximum when the pH of the meat was around 5.

So far, few studies have been directed towards the alteration of proteins and lipids during the freezing and storage of bovine muscle. Sizov (1956) found that the solubility of the total salt-soluble proteins in bovine muscle was decreased to 60% during a storage period of 22 days at −14°C. When bovine muscle was stored at −20°C for 5 weeks, Kronman *et al.* (1960) noted a reduction in the amount of extractable sarcoplasmic proteins.

[a] Postdoctoral research associate.

Smorodintsev (1943 a,b) failed to find any change in protein solubility over a 2-month period at −8°C. Ramsbottom (1947) reported an increase in both peroxide value and free fatty acids in frozen stored beef but no change in total lipid was noted. Guerrant *et al.* (1953) showed an increase in peroxide value and free fatty acids of beef stored at −12.2, −17.8 and −28.9°C.

The initiation of this preliminary study was prompted by the lack of detailed data on the chemical alterations of frozen bovine muscle. The storage temperature of −4°C was selected so that extensive deterioration would occur within a few months. According to Riedel (1957), beef with a moisture content of 74% would have an ice content (by weight) of about 70% with a storage temperature of −4°C.

MATERIALS AND METHODS

Muscle samples

Muscle samples, each weighing about 200 g, were cut from the round of a beef carcass after a 7-day aging period at around 4°C. Muscle from only one animal was used to insure compositional uniformity of experimental samples. All samples were devoid of extramuscular adipose tissue. After the samples were wrapped in Saran film, they were frozen and held at −4°C for various periods up to 8 weeks. Zero freezing time was considered as approximately 18 hr of muscle storage at −4°C. At least two randomized muscle samples were used for each determination at one storage time.

Total nitrogen of muscle

The total nitrogen of muscle samples was determined by a micro-Kjeldahl procedure (AOAC, 1965). Protein content of muscle was calculated by multiplying the nitrogen content by 6.25.

Protein content in muscle fractions

The amount of nitrogen in various protein fractions of muscle was determined by the biuret method of Snow (1950). A factor of 6.25 was used to convert nitrogen to protein content.

Protein extraction and fractionation

Muscle, ground in a Hobart grinder with a plate having 0.5 cm holes, was mixed with powdered CO_2 dry ice (20 g of ground muscle to 100 g of dry ice) in a Waring blendor to disrupt extensively the muscle fibers. This dry ice procedure was used to prevent protein insolubilization during tissue maceration. After thawing at 25°C, 2 g of macerate were mixed in a mortar with 100 ml of Na phosphate-KCl buffer, pH 7.2, 1.0 μ (4°C). The muscle slurry at 4°C was agitated for 2 hr with a magnetic stirring

rod. The mixture was centrifuged for 20 min at 1500 × G. The supernatant was used to determine the amount of total extractable protein (TEP).

Soluble actomyosin in the above-mentioned supernatant was precipitated by diluting an aliquot of the supernatant with water to produce an ionic strength of 0.25. The mixture, after standing for 16 hr at 4°C, was centrifuged at 2100 × G for 30 min. The precipitated protein fraction was then washed with distilled water and the mixture was centrifuged at 2100 × G to obtain purified actomyosin. The precipitated actomyosin was dissolved in 1 *N* NaOH to determine the nitrogen content by the biuret method.

Sarcoplasmic protein was extracted from macerated muscle (disrupted by the dry ice method) with 0.05 μ phosphate buffer (pH 7.2). For each sample, 2 g of macerate were blended with 100 ml of phosphate buffer in a Waring blendor for 3 to 4 min and the suspension was centrifuged at 1500 × G for 20 min. The residue was re-extracted twice with phosphate buffer. The amount of sarcoplasmic protein in an aliquot of combined supernatants was measured by the biuret method.

Disc electrophoresis of proteins

The disc electrophoresis technique, described by Davis (1964), was used to separate protein fractions extracted from unfrozen and frozen muscle samples. This electrophoretic procedure involved the separation of the proteins in tubes containing polyacrylamide gel with Tris buffer, pH 8.1. A current of 5 ma per tube was applied for about 1.5 hr while the tubes were held in a refrigerated cabinet at 6°C. In each tube, 0.2 ml of large pore gel were layered on 1.8 ml of small pore gel. An aliquot of protein solution containing about 0.2 mg protein was applied on top of each upper gel.

After completing an electrophoretic run, the proteins in the polyacrylamide gel were stained by immersing the gel in 1% amido black (Schwarz) dye. After electrophoretically destaining each gel in 7% acetic acid, the protein fractions appeared as dark bands. A Joyce chromoscan, made by the Joyce-Loebl and Company, Ltd., England, was used to make tracings of the polyacrylamide gel patterns. An interference filter with a maximum transmittance at 660 mμ was used.

Prior to electrophoresis, sarcoplasmic protein solutions and thaw drip were mixed with sucrose to produce a 15% sucrose solution. The small pore gel used for these samples contained 1.5% acrylamide as recommended by Davis (1964).

Preliminary experiments indicated that myofibrillar proteins could not be separated by gel electrophoresis without adding urea to the protein solutions and polyacrylamide gel. Urea was added to each solution, containing myofibrillar proteins, to bring the concentration to 8 *M*. The concentration of urea in the acrylamide gel solutions (5% acrylamide) was 4 *M*.

Measurement of drip volume

Prior to drip estimation, each muscle sample in the frozen state was ground in a Hobart grinder with a plate having openings of 0.5 cm in diameter. The drip from ground muscle, either thawed at 25°C or cooked at 70°C for 60 min in a special centrifuge tube, was obtained by the centrifugal method of Miyauchi (1962). All samples were centrifuged at 1000 × G for 10 min.

Extraction of lipids

Lipids were extracted from unfrozen or thawed muscle by the method of Bligh *et al.* (1959). The chloroform in the lipid extract was evaporated under vacuum using a rotary evaporator with a 500-ml flask in a water bath at 30°C.

The total lipid content of a muscle sample was determined by using 50-ml aliquots of the lipid extract. When the chloroform was removed by holding the extract at 60°C under vacuum, the oily residue was dried at 70°C for 3 hr under vacuum.

Lipid analyses

Total cholesterol in aliquots of lipid extracts was determined by the method of Tu *et al.* (1967).

Phosphorus content of lipid extracts was estimated by the method of Morrison (1964). The phospholipid content was calculated by multiplying the phosphorus content by 25.

Concentration of free fatty acids in an aliquot of lipid extract was determined by the method of Mosinger (1965). Palmitic acid was used as the standard.

The method of Dyer *et al.* (1956) was used for the measurement of the peroxide value of muscle lipid. The thiobarbituric acid (TBA) number was determined by the method of Yu *et al.* (1957).

Thin layer chromatography

Aliquots containing around 400 μg lipids in chloroform were spotted on layers of silica gel with 10% $CaSO_4$ (Adsorbosil-1; Applied Science Laboratories) having a thickness of 0.5 mm. The chromatograms were developed with petroleum ether-ether-acetic acid (90/10/1) solvent system for the separation of the neutral lipids. Lipid spots were detected by spraying 50% H_2SO_4 on the plates and heating at 110°C for about 1 hr.

RESULTS AND DISCUSSION

Protein solubility of frozen stored muscle

The solubility data of protein fractions from unfrozen and frozen stored bovine muscle are presented in Table 1. Total extractable protein (TEP) from unfrozen bovine muscle was estimated to be about 91%. Recently McIntosh

Table 1. Influence of frozen storage at −4°C on the solubility of proteins in bovine muscle.

Frozen storage time (weeks)	Soluble protein (% of total protein)		
	Total extractable protein	Actomyosin	Sarcoplasmic protein
Unfrozen	90.99 (2.35) [1]	51.60 (2.04)	31.96 (1.93)
0	85.44 (2.12)	51.40 (2.54)	28.55 (1.63)
1	74.25 (3.00)	43.00 (1.37)	27.68 (0.61)
2	62.14 (2.29)	30.18 (1.07)	26.92 (0.92)
4	59.39 (2.95)	26.66 (2.24)	23.92 (1.12)
5	59.34 (0.92)	25.90 (0.71)	20.20 (1.53)
6	55.06 (2.34)	22.50 (1.78)	16.39 (1.07)
8	50.84 (1.78)	17.15 (1.58)	12.82 (1.42)

[1] Mean and standard deviation in brackets.

(1967) reported a total extractable nitrogen value of 71% for beef muscle aged for 6 days at 4°C. According to Saffle *et al.* (1964), the average TEP of unfrozen cow and bull meat was about 38%. These lower TEP values may be attributed to the surface denaturation and aggregation of muscle proteins during the protein extraction procedure. Preliminary experiments in the present study indicated that with maceration of muscle and protein extraction with the phosphate buffer in a Waring blendor, the TEP value for bovine muscle was between 45 and 50%.

Blending ground bovine muscle with powdered dry ice for tissue fiber disruption obviated the extensive insolubilization of the muscle proteins. As reported in Table 1, the % TEP of frozen muscle became progressively less as storage time at −4°C increased. At zero storage time (18 hr in the freezer at −4°C), about 85% of the total protein was extractable; and after 8 weeks of storage, the TEP was down to about 51%. The % TEP declined most rapidly during the first 2 weeks of frozen storage.

Saffle *et al.* (1964) reported that protein extractability of 48-hr post-rigor bovine meat decreased about 9% during frozen storage at −34°C for 2 days. According to Khan *et al.* (1963), the % total extractable nitrogen of chicken breast muscle stored at −4°C decreased from about 88 to approximately 56 over a 50-week period.

In this study, actomyosin has been classified as the myofibrillar protein fraction insoluble in KCl-phosphate buffer (pH 7.2) at an ionic strength of 0.25 (Khan, 1962). As shown in Table 1, the soluble actomyosin content of unfrozen bovine muscle was estimated to be about 52%. Aberle *et al.* (1966) reported that the fibrillar protein nitrogen extracted from bovine semitendinosus muscle (7 days post-mortem) was about 56% of the total nitrogen. The average fibrillar protein N of longissimus dorsi from 20 animals was calculated by Hegarty *et al.* (1963) as 62% of the total nitrogen in muscle.

When bovine muscle was frozen for 18 hr (zero storage time), the actomyosin solubility was not altered significantly. However, further frozen storage of muscle up to 8 weeks caused a considerable decline in soluble actomyosin. Over an 8-week frozen storage period, the soluble actomyosin content of bovine muscle decreased about 70%. The % soluble actomyosin decreased most rapidly in the first 2 weeks of frozen storage as did the % TEP. Khan *et al.* (1963) found that the actomyosin in both chicken breast and leg muscles decreased considerably during frozen storage at −4°C for 50 weeks.

In this study, the sarcoplasmic protein fraction of bovine muscle consisted of proteins and peptides which were soluble in phosphate buffer with an ionic strength of 0.05. Table 1 shows that the sarcoplasmic protein was about 32% of the total bovine muscle protein. The % sarcoplasmic protein nitrogen extracted from 7-day post-mortem longissimus dorsi and semitendinosus muscles was estimated by Aberle *et al.* (1966) to be 23.3 and 20.8, respectively. These researchers did not define sarcoplasmic protein nor did they indicate the method of fractionation. However, they reported that the nonprotein nitrogen values for 7-day aged longissimus dorsi and semitendinosus muscles were 14.8 and 14.1%, respectively, of the total nitrogen.

Hegarty *et al.* (1963) defined sarcoplasmic protein nitrogen as the nitrogen extracted from muscle by a low ionic strength buffer (0.05 μ) minus the nonprotein nitrogen. They reported that, for unfrozen longissimus dorsi muscles from 20 yearling bulls, the average percentage sarcoplasmic protein nitrogen and percentage nonprotein nitrogen were 31 and 6.5, respectively.

Data in Table 1 indicate that during frozen storage of bovine muscle for 8 weeks at −4°C, the sarcoplasmic protein content decreased from about 29 to 13%. The sarcoplasmic protein solubility was lost slowly and progressively throughout the entire storage period. With 3-hr post-mortem bovine muscle, Kronman *et al.* (1960) found that the freezing of the muscle caused a considerable decrease in the amount of water-extractable protein.

Disc electrophoresis of soluble proteins

The electrophoretic separation of sarcoplasmic proteins, using polyacrylamide gel, was used to follow the protein alteration due to frozen storage of muscle. Fig. 1 shows the electrophoretic pattern and densitometric tracing for sarcoplasmic protein extracted from unfrozen bovine muscle. Fourteen distinct dye-stained bands, including at least 8 major deep-colored bands, were observed. Aberle *et al.* (1966), using starch gel electrophoresis, reported that 15 bands could be detected in the electrophoretic patterns of sarcoplasmic protein from unaged and aged (up to 336 hr) bovine muscles. As post-mortem aging of muscle proceeded, they found that the bands became more discrete and a few bands changed in color intensity.

During the frozen storage of bovine muscle, some sarcoplasmic proteins were altered to such an extent that they did not migrate down into the polyacrylamide gel during electrophoresis. Fig. 2 shows the electrophoretic pattern and tracing of sarcoplasmic protein extracted from muscle

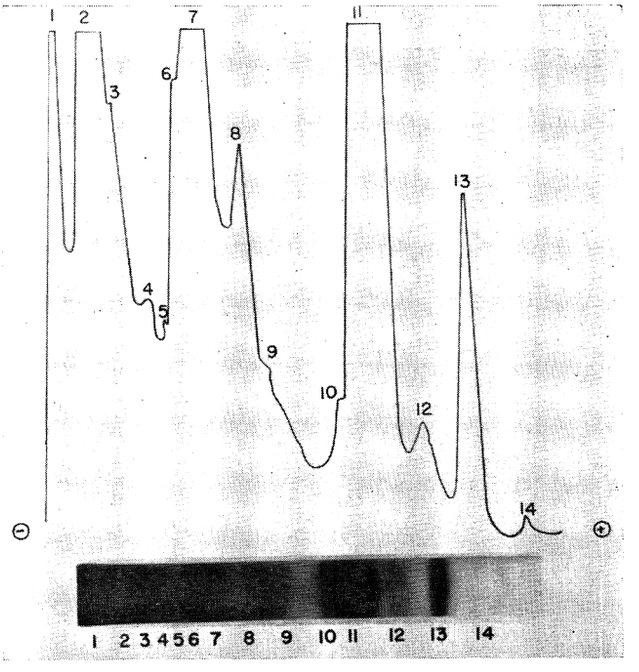

Fig. 1. Electrophoretic pattern and tracing of sarcoplasmic protein from unfrozen bovine muscle.

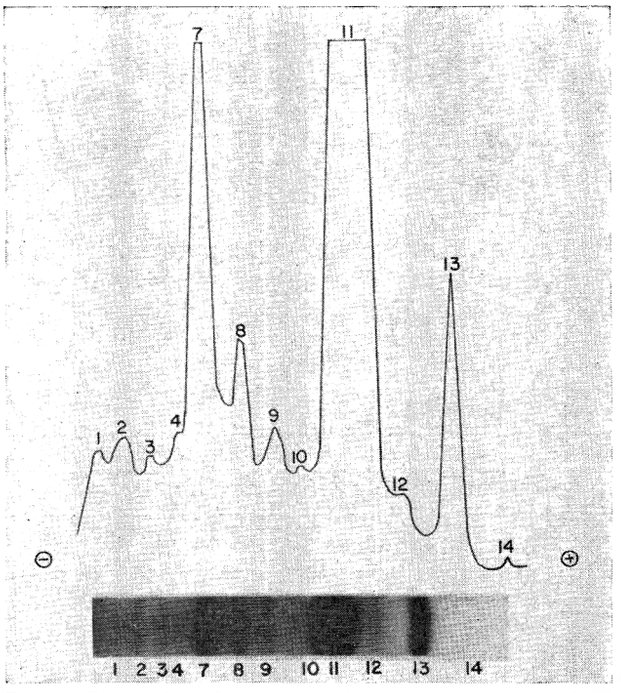

Fig. 2. Electrophoretic pattern and tracing of sarcoplasmic protein from bovine muscle stored for 2 weeks at —4°C.

held in frozen storage for 2 weeks. In comparison to the bands shown in Fig. 1 for unfrozen muscle, bands 5 and 6 were absent and the bands 1, 2 and 3 were much less intense in color. Apparently, specific sarcoplasmic proteins were insolubilized readily within the 2-week storage period of muscle. As shown in Fig. 3, bands 1, 4, 5, 6 and 10 were missing from the pattern for sarcoplasmic protein from muscle stored for 8 weeks. Moreover, the color intensities of bands 7, 11 and 13 in Fig. 3 were considerably lower than those in Fig. 2.

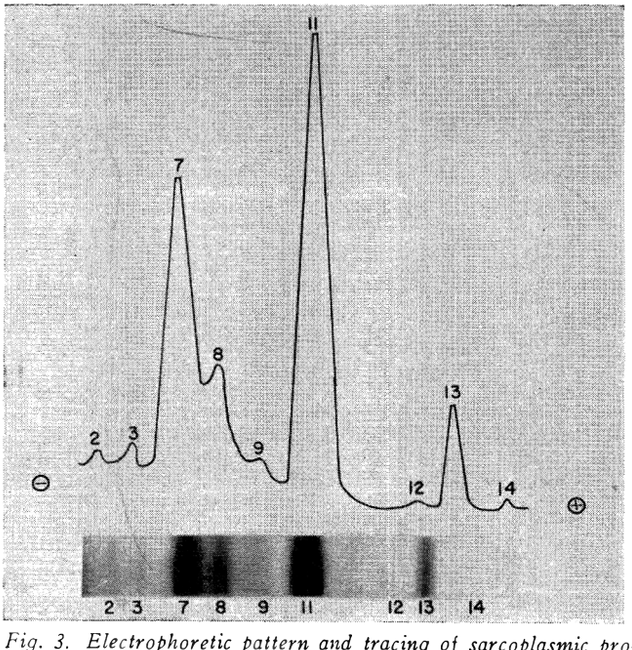

Fig. 3. Electrophoretic pattern and tracing of sarcoplasmic protein from bovine muscle stored for 8 weeks at —4°C.

In this study, urea was added to bovine actomyosin to disrupt the high-molecular weight, heterogeneous complexes and solubilize the fibrillar proteins for electrophoretic separation. Using urea-polyacrylamide gel electrophoresis, urea-treated actomyosin from unfrozen muscle was separated into 6 distinct bands (Fig. 4). Bands 1 and 4 were stained strongly and thus probably represent the major myofibrillar proteins in the actomyosin fraction. Certainly disc electrophoretic analysis showed that urea-treated bovine actomyosin prepared by the method of Khan (1962) was heterogeneous.

Actomyosin, extracted from bovine muscle previously held in frozen storage for 2 weeks, separated into four bands (Fig. 5) with bands 2 and 6 being absent. With muscle stored for 8 weeks, the actomyosin was separated into three bands with 2, 3 and 6 missing (Fig. 6). The presence of the remaining light-colored bands, 1, 4 and 5, indicated that the major myofibrillar proteins of bovine muscle were not completely insolubilized during the frozen storage of muscle for 8 weeks.

Neelin *et al.* (1964) extracted myofibrillar proteins from minced chicken muscle by 8 *M* urea solution for starch gel electrophoresis. Using a urea-containing buffer (pH 7.5) for electrophoresis, six myofibrillar protein bands and three bands attributed to myogen proteins were resolved. All of the myofibrillar proteins were negatively charged while the myogen proteins were charged positively. Fujimaki *et al.* (1965) were able to separate rabbit actomyosin into four fractions using TEAE cellulose chromatography.

Volume and protein content of drip

The determination of the amount of drip from bovine muscle has been used as a measure of the water-binding

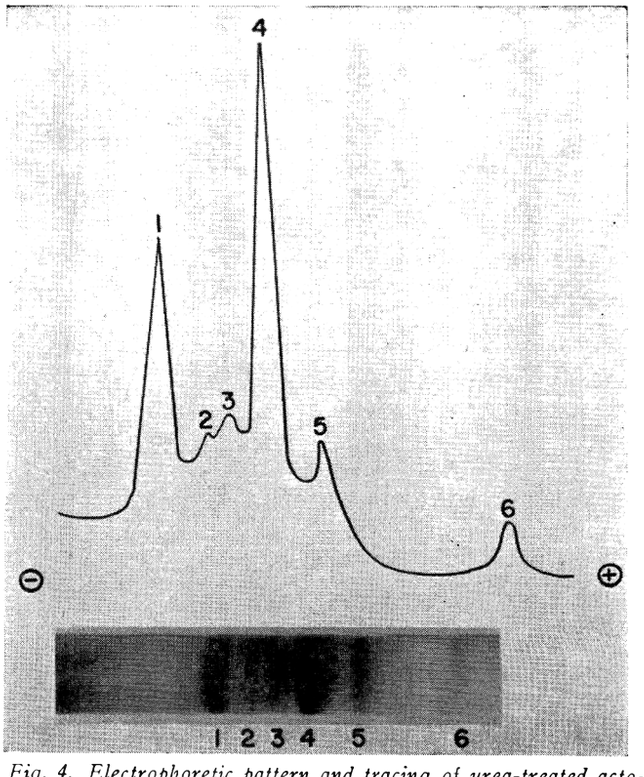

Fig. 4. Electrophoretic pattern and tracing of urea-treated actomyosin from unfrozen bovine skeletal muscle.

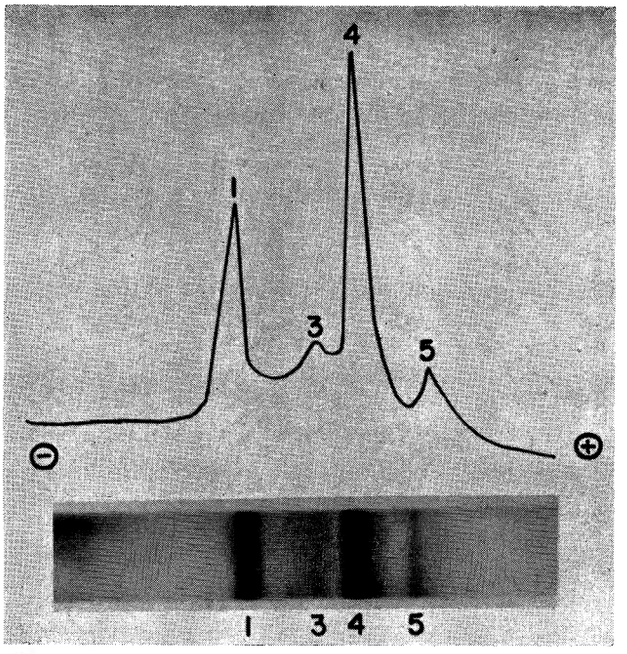

Fig. 5. Electrophoretic pattern and tracing of urea-treated actomyosin from bovine skeletal muscle stored for 2 weeks at −4°C.

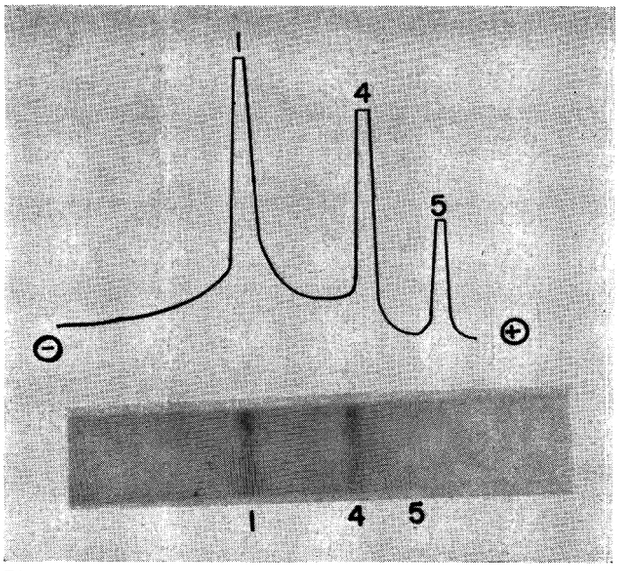

Fig. 6. Electrophoretic pattern and tracing of urea-treated actomyosin from bovine skeletal muscle stored for 8 weeks at −4°C.

capacity (Wierbiki *et al.*, 1957). Table 2 presents data on the amount of drip obtained upon the thawing of muscle, previously stored up to 8 weeks at −4°C. Even with a brief freezing time of 18 hr (zero storage time), the amount of drip from thawed muscle was about twice as large as that from unfrozen muscle. Thus freezing per se had an influence on the amount of drip from bovine muscle. As storage time progressed up to 8 weeks, the volume of drip per 100 g of muscle increased up to 24 ml. Obviously, the water-binding capacity of bovine muscle was reduced considerably during the 8-week storage at −4°C.

The increase of drip with increasing the frozen storage period is in accord with the findings of Pearson *et al.* (1950) and Wierbiki *et al* (1957) for stored bovine muscle. The pH of the drip did not change with the storage period as shown in Table 2. The pH of all drip samples ranged from 5.5 to 5.7.

The loss of proteins, mostly sarcoplasmic protein, in the drip of previously-frozen bovine muscle has been reported by Howard *et al.* (1960). The protein content of drip, calculated as percentage of total muscle protein, increased from about 5.86 for unfrozen muscle to 17.03 for muscle frozen for 8 weeks (Table 2). The greatest increase in the amount of protein occurred during the first 4 weeks of storage; thereafter, the increases were comparatively insignificant.

As shown in Table 3, the amount of cook drip from unfrozen and frozen muscle was much higher than the volume of thaw drip from comparable samples (Table 2). Table 3 shows that upon freezing bovine muscle for 18 hr (zero storage time) the volume of cook drip was twice as much as the cook drip obtained from unfrozen muscle. From 0 up to 8 weeks of storage, a steady but slow increase of the amount of drip was obtained to the extent of about 42 ml per 100 g muscle. The protein content in thaw drip was much higher than that in cook drip for each storage time.

The pH range of cook drip was slightly higher than that for thaw drip. This observation is in agreement with the results of Hamm *et al.* (1960) and Deatherage *et al.* (1960). These investigators indicated that an increase of the basic groups on the protein molecules occurred upon heating of muscle. The pH of cook drip increased from 5.8 for unfrozen muscle to 6.1 for muscle stored for 8 weeks.

When the centrifuge drip from the unfrozen muscle macerate was subjected to polyacrylamide gel electropho-

Table 2. Influence of frozen storage at −4°C on the thaw drip from bovine muscle.

Frozen storage time (weeks)	Drip (ml/100 g muscle)	Protein in drip		
		g/100 g muscle	% total protein	pH of drip (range)
Unfrozen	7.3 (1.0) [1]	1.15 (0.10)	5.86 (0.51)	5.5–5.6
0	15.9 (1.0)	1.31 (0.20)	6.72 (1.02)	5.5–5.6
1	18.5 (1.1)	1.67 (0.09)	8.50 (0.46)	5.5–5.6
2	19.5 (1.0)	2.38 (0.26)	12.10 (1.32)	5.5–5.6
4	22.0 (1.6)	3.26 (0.13)	16.58 (0.66)	5.5–5.7
5	22.8 (1.0)	3.30 (0.30)	16.80 (1.53)	5.5–5.6
6	23.5 (1.5)	3.31 (0.39)	16.82 (1.98)	5.5–5.6
8	24.0 (1.1)	3.35 (0.24)	17.03 (1.22)	5.5–5.6

[1] Mean and standard deviation in brackets.

Table 3. Influence of frozen storage at −4°C on the cook drip from bovine muscle.

Frozen storage time (weeks)	Cook drip (ml/100 g muscle)	Protein in cook drip		pH of cook drip (range)
		g/100 g muscle	% of total protein	
Unfrozen	16.4 (1.6) [1]	0.16 (0.01)	0.80 (0.05)	5.8
0	34.8 (0.7)	0.44 (0.07)	2.19 (0.36)	5.9
1	39.3 (0.9)	0.56 (0.06)	2.86 (0.30)	5.9
2	40.0 (0.4)	0.60 (0.05)	3.08 (0.25)	5.8–5.9
4	40.1 (0.8)	0.61 (0.04)	3.13 (0.20)	5.8–5.9
5	40.8 (1.0)	0.64 (0.06)	3.27 (0.30)	5.8–6.0
6	41.3 (1.4)	0.69 (0.01)	3.51 (0.05)	5.8–6.0
8	41.9 (1.3)	0.67 (0.01)	3.49 (0.05)	6.0–6.1

[1] Mean and standard deviation in brackets.

resis, the resulting pattern (Fig. 7) containing 13 bands was similar to the electrophoretic pattern (Fig. 1) for sarcoplasmic proteins extracted from unfrozen bovine muscle. As shown in Fig. 8, the electrophoretic pattern of thaw drip from 8-week frozen stored muscle was devoid of bands 4, 5, 8 and 13, but contained a new minor band X. In general, the major bands in this pattern were not as intense blue as those in the pattern (Fig. 7) for drip from unfrozen muscle.

Lipid alterations during frozen storage of muscle

The concentrations of lipids from unfrozen and frozen stored muscle are reported in Table 4. The total lipid, extracted with the chloroform-methanol solvent system, changed slightly in concentration over the 8-week storage period. The concentration of total cholesterol in unfrozen bovine muscle was between 62 and 64 mg per 100 g muscle. Tu *et al.* (1967) found that the average total cholesterol content for 5 types of bovine muscles was about 58 mg percentage. During frozen storage, no significant change in the total cholesterol of the bovine muscle was evident. Apparently, cholesterol in bovine muscle was resistant to deterioration during frozen storage.

As shown in Table 4, phospholipid represented about 28% of the total lipid from unfrozen muscle. On a wet muscle basis, the phospholipid content was approximately 1%. Hornstein *et al.* (1961) also found that bovine muscle contained about 1% phospholipid. A considerable decrease in the phospholipid content was evident as the frozen storage time of muscle progressed. A slight decline in the phospholipid content was evident during the first 2 weeks of frozen storage, then the phospholipid loss was high between the 2nd and 5th weeks of storage. Probably, the drop in phospholipid concentration can be accounted for by the enzymic hydrolysis of phospholipid during the frozen storage of the muscle. Olley *et al.* (1960) indicated that hydrolysis of phospholipids occurred during frozen

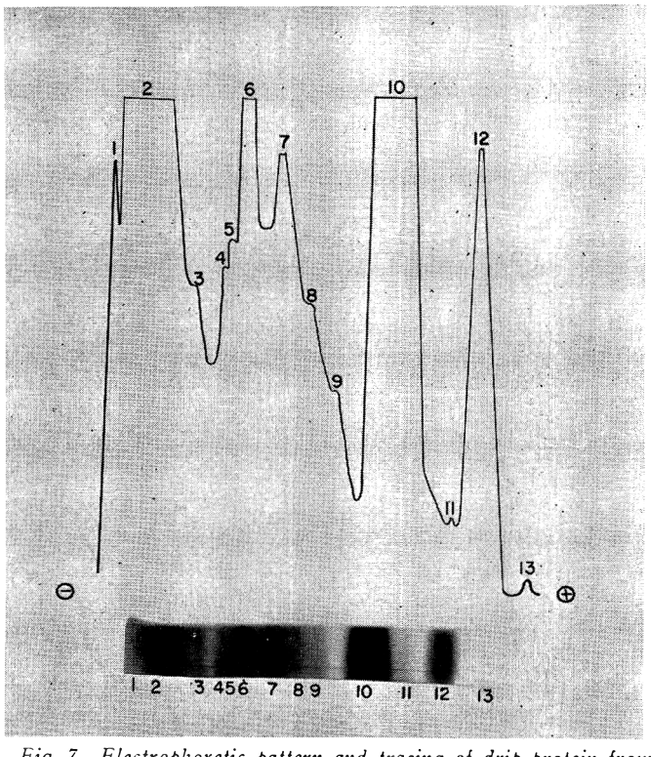

Fig. 7. Electrophoretic pattern and tracing of drip protein from unfrozen bovine muscle.

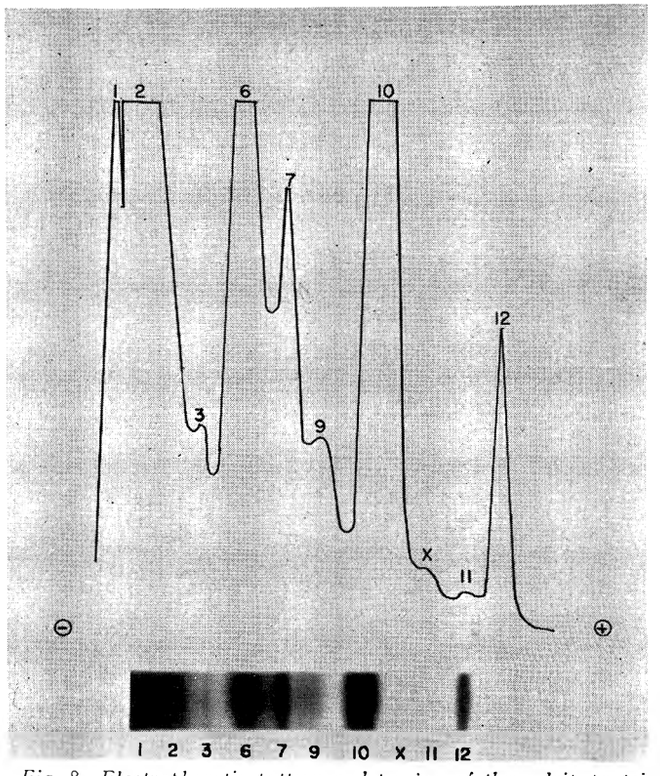

Fig. 8. Electrophoretic pattern and tracing of thaw drip protein from bovine muscle stored for 8 weeks at −4°C.

Table 4. Lipids from unfrozen and frozen stored bovine muscle.

Frozen storage time (weeks)	Total lipid	Total cholesterol		Phospholipid		Free fatty acids	
	g/100 g muscle	% of total lipid	mg/100 g muscle	% of total lipid	mg/100 g muscle	% of total lipid	mg/100 g muscle
Unfrozen	3.75 (0.14) [1]	1.7 (0)	63 (1)	28.8 (1.0)	1079 (31)	1.6 (0)	61 (2)
1	3.61 (0.23)	1.8 (0)	64 (1)	28.3 (0.9)	1022 (32)	2.9 (0)	103 (0)
2	3.60 (0.21)	1.8 (0.1)	63 (2)	27.8 (1.1)	1001 (38)	2.9 (0)	106 (0)
4	3.60 (0.21)	1.7 (0)	62 (1)	22.3 (1.0)	803 (36)	7.9 (0)	284 (1)
5	3.58 (0.19)	1.8 (0)	64 (1)	20.9 (1.3)	752 (45)	8.5 (0.1)	304 (4)
6	3.48 (0.24)	1.8 (0)	63 (1)	19.6 (0.6)	698 (20)	8.8 (0.3)	308 (12)
8	3.38 (0.07)	1.8 (0)	62 (0)	19.5 (0.5)	663 (15)	9.1 (0.1)	308 (5)

[1] Mean and standard deviation in brackets.

storage of cod muscle with the formation of free fatty acids and water-soluble decomposed phospholipids.

Lipid from unfrozen bovine muscle contained about 1.6% free fatty acids (FFA) (Table 4). During frozen storage, a marked increase in the FFA content of frozen muscle occurred between the 2nd and 5th weeks of storage. At the 8-week storage time, the FFA content of the lipid reached 9.1%. These results indicate a close relationship between the loss of phospholipids and the rise in FFA content of frozen stored muscle.

The amount of FFA originating from phospholipids in frozen bovine muscle can be estimated by using the data in Table 4. If the average molecular weight of a fatty acid of muscle phospholipid is considered to be 300 (Olley *et al.*, 1960), then a total of 600 g of FFA could be formed from 775 g of phospholipid. With 100 g of frozen bovine muscle, the total possible amount of FFA from decomposed phospholipid was estimated to be 321 mg but only 247 mg FFA accumulated during the 8-week storage period. Thus 130% FFA was calculated as the theoretical amount derived from phospholipids due to hydrolysis. A value greater than 100% indicates that phospholipid was broken down to water-soluble phosphate esters by phospholipase C (Olley *et al.*, 1962).

The FFA, which were liberated during frozen storage, may have interacted with bovine myofibrillar proteins to render them insoluble. Dyer *et al.* (1956) indicated a relationship between fish "actomyosin" insolubilization and FFA release in frozen fish muscle. Within recent years, many research studies have been directed to determine the validity of the postulation that FFA-protein interactions are responsible for protein insolubilization in frozen fish (Dyer *et al.*, 1961; Connell, 1964; Fennema *et al.*, 1964; Love, 1966).

Several investigators (Callow, 1952; DuBois *et al.*, 1940; Privett, 1954; Ramsbottom, 1947) have reported that oxidative deterioration of lipids occurred during frozen storage of meat. The peroxide value (PV) and thiobarbituric acid (TBA) number are used often for numerically assessing the degree of lipid autoxidation.

The PV and TBA numbers for lipid from unfrozen and frozen stored bovine muscle are presented in Table 5. The PV of lipid from unfrozen muscle was 3.5, a value indicating the presence of a small amount of peroxide in the lipid. Within a 2-week frozen storage period, the PV of muscle lipid rose to 44.7 and then, with subsequent storage periods, the values dropped until a constant value of about 7 was reached for 6 and 8 weeks of storage. This rise and decline of PV for muscle lipid during frozen muscle storage may be explained by accumulation of hydroperoxides in the lipid fraction and then (1) subsequent hydroperoxide decomposition and/or (2) hydroperoxide interaction fith muscle proteins (Desai *et al.*, 1963; Naryan *et al.*, 1964).

The TBA number for lipid from unfrozen muscle was about 0.02 (Table 5). During frozen storage of muscle, the TBA number increased up to 0.27 for the 8-week storage period. TBA numbers of slices of lamb longissi-

Table 5. Lipid deterioration in frozen bovine muscle.

Frozen storage time (weeks)	Peroxide value (meq thiosulfate/kg lipid)	TBA number (optical density range)
Unfrozen	3.5 (0.2) [1]	0.02–0.03
1	16.2 (2.2)	0.09–0.10
2	44.7 (3.2)	0.11–0.12
4	13.2 (0.1)	0.13–0.14
5	7.8 (0.1)	0.18–0.19
6	7.2 (0.5)	0.25–0.26
8	7.1 (0)	0.27

[1] Mean and standard deviation in brackets.

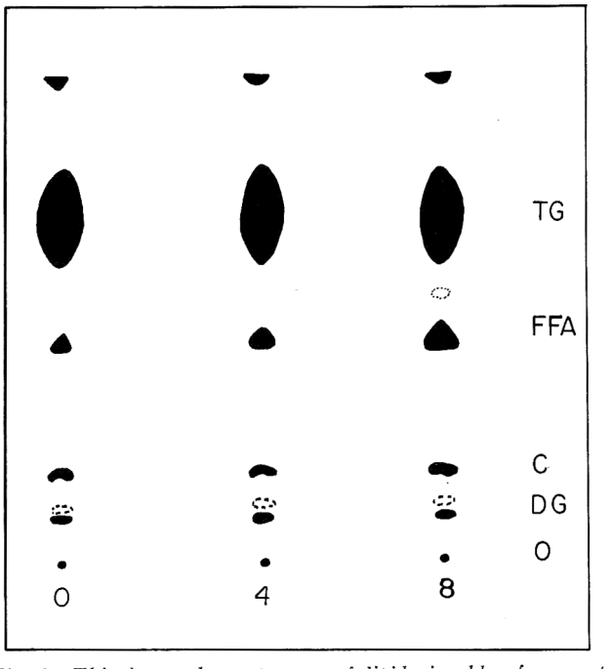

Fig. 9. Thin layer chromatogram of lipids in chloroform-methanol extracts of bovine muscle stored at —4°C for 0, 4 and 8 weeks. TG = triglyceride, FFA = free fatty acids, C = cholesterol, DG = diglyceride, O = origin.

mus dorsi, stored at −18°C, rose from 0.13 (0 days of storage) to 1.93 for 9.5 months of storage (Keskinel *et al.*, 1964).

Thin layer chromatography (TLC) was employed to fractionate the muscle lipid into steroid esters (at solvent front), triglycerides (TG), free fatty acids (FFA), cholesterol (C), and diglycerides (DG) in order to detect any changes in these lipid fractions during muscle storage at −4°C. TLC separation of lipids from muscle stored for 0, 4 and 8 weeks are shown in the chromatogram of Fig. 9. The enlargement of the FFA spot was evident with an increase in frozen storage time. With lipid from 8-week stored muscle, a small spot was present between the TG and FFA spots. No other spot size changes or new spots were apparent in the chromatogram.

CONCLUSIONS

With the knowledge that extensive protein and lipid alterations occur in frozen, stored bovine muscle at −4°C, further studies should be designed to assess cumulative changes in frozen bovine muscle at storage temperatures commonly used by the frozen food industry.

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JEAN M. HOWE, N. W. THOMAS, P. B. ADDIS and M. D. JUDGE
Department of Animal Sciences, Purdue University, Lafayette, Indiana 47907

Temperature Acclimation and Its Effects on Porcine Muscle Properties in Two Humidity Environments

SUMMARY—The effects of temperature and humidity on post-mortem and associated muscle properties during growth of "stress susceptible" pigs were evaluated. Exposure to ambient temperatures of 32 and 21°C for alternating 3-day periods caused rapid post-mortem glycolysis, high percent light reflectance, and increased light to dark fiber ratios in the longissimus dorsi muscle as compared to constant (27°C) temperature, but only in moderate (38–42% relative) humidity environments. The above events due to temperature acclimation were masked when the humidity was low (17–23% relative). Humidity effects that were independent of temperature acclimation resulted in high percent light reflectance and high muscle temperature in the post-mortem muscle of pigs reared in low humidity. No significant differences were found in lactic dehydrogenase or succinic dehydrogenase enzyme activities of longissimus dorsi or gluteus medius muscles.

INTRODUCTION

IN RECENT YEARS numerous biochemical aspects of post-mortem changes in porcine muscle have been established (Briskey, 1964; Briskey *et al.*, 1966). Research has been focused on the chemical and physical muscle characteristics associated with the development of the pale, soft, exudative (PSE) condition (Briskey, 1964). The incidence of PSE muscle depends on many factors such as breed (Sayre *et al.*, 1963a; Judge *et al.*, 1959), muscularity (Topel *et al.*, 1967; Addis *et al.*, 1967a), ante-mortem handling (Briskey *et al.*, 1959; Sayre *et al.*, 1963b; Kastenschmidt *et al.*, 1964; Briskey *et al.*, 1965), nutrition (Briskey *et al.*, 1959, 1960; Sayre *et al.*, 1963b; Lawrie, 1966) and others. However the effects of environmental conditions during growth have not been thoroughly evaluated.

Thomas *et al.* (1966) reported that longissimus dorsi muscle of pigs reared in fluctuating temperatures (29 and 18°C) was inferior in structure to that of pigs reared in 29 or 18°C constant temperatures if the relative humidity was 30%. The findings of Addis *et al.* (1967b) indicated that temperature acclimation in pigs intensifies the correlation of physiological stress responses with post-mortem muscle properties. Humidity level during growth may also influence post-mortem events in light of the tendency for pigs reared in 85% relative humidity to have longissimus dorsi muscles that are superior in color and gross morphology (Addis *et al.*, 1967a) and lower in shear resistance (Thomas *et al.*, 1966) than those of pigs reared in 30% relative humidity. The above observations indicate that the quality of pork may be improved or made more uniform by use of controlled rearing environments but they do not explain the effects, if any, of such environments on muscle differentiation or metabolic capacity.

This investigation was conducted to study the effects of various combinations of environmental temperature and humidity during growth on certain physico-chemical properties of porcine muscle.

EXPERIMENTAL

Animals and growing environment

Forty "stress-susceptible" (Judge *et al.*, 1967) Poland China barrows were randomly allotted and reared in psychrometric chambers under the humidity and temperature conditions described in Table 1. The time required to change from one temperature to the other in the chambers housing the groups subjected to fluctuating temperatures was 30 min. Both humidity and temperature were continuously recorded. The respiration rate and body temperature of each animal were measured daily by counting flank movements and with standardized rectal thermometers. Twenty-four hr prior to sacrifice, the temperature was adjusted to 27°C in both chambers to eliminate immediate-ante-mortem temperature effects. The animals were stunned by captive bolt pistol with minimal excitement prior to removal from their pens.

Post-mortem changes

The internal temperature (bimetallic testing thermometer accurate to within 0.005°C), surface pH (probe

Table 1. Experimental groups, environmental conditions and weight gain of pigs reared in psychrometric chambers.

Group	Ambient temperature (°C)	Relative humidity (%)	Animals (no.)	Days (no.)	Weight gain[4,5] (kg/da)
H_1T_1[1,2]	27	42	8	73	0.69
H_1T_2	32, 21[3]	38	8	73	0.56
H_2T_1	27	17	12	134	0.60
H_2T_2	32, 21[3]	23	12	134	0.54

[1] Designates relative humidity.
H_1 = Moderate humidity (38–42% r.h., ave. 10 g/cu m of air).
H_2 = Low humidity (17–23% r.h., ave. 5 g/cu m of air).
[2] Designates temperature.
[3] Alternating 3-day periods at each temperature.
[4] Significant humidity effect ($P < .01$; groups H_1T_1 and H_1T_2 vs. groups H_2T_1 and H_2T_2).
[5] Significant temperature effect ($P < .01$; groups H_1T_1 and H_2T_1 vs. groups H_1T_2 and H_2T_2).

electrode) and surface reflectance (Bausch and Lomb "Spectronic 20" with reflectance attachment at 525 mμ) were observed at 15-min intervals up to 45 min and at 24-hr post-mortem on freshly cut surfaces of the longissimus dorsi muscles. Reflectance values were adjusted, using a magnesium carbonate block as 100% reflectance reference.

Histochemical observations

Samples of longissimus dorsi and gluteus medius muscle were cut into cubes (10 × 5 × 5 mm) and placed in 10% formal-calcium for 18–24 hr. Sixteen-micron sections were cut in a cryostat at −20°C and stained with Sudan black B (Beecher *et al.*, 1965). The light to dark fiber ratios were determined in 9 bundles per sample.

Enzyme assays

Within 5 min of the beginning of exsanguination, muscle samples were obtained and immediately frozen in liquid nitrogen. Homogenates were prepared by grinding frozen tissues in 2 vol (w/v) of 0.1*M* phosphate buffer pH 7.4 in an ice-jacketed Servall Omni Mixer for 30 min. Homogenates were then centrifuged for 30 min at 27,000X G (2°C) and the supernatant fluids were assayed for total lactic dehydrogenase (LDH) activity.

Total lactic dehydrogenase activity was determined by recording the change in extinction at 340 mμ resulting from the oxidation of NADH in pH 7.4 buffered solution containing $8.4 \times 10^{-4}M$ sodium pyruvate (Bergmeyer *et al.*, 1963). Measurements were made with a ratio-recording Beckman DB spectrophotometer at 25°C. One unit of LDH was defined as the amount of enzyme which changes the absorbance of NADH at 340 mμ by 0.0001 in 1 min in a 3-ml assay mixture and at 24–27°C. Protein concentration of the extract was determined by a biuret method (Layne, 1957). Results are reported as units of LDH per milligram tissue protein.

One percent (w/v) homogenates of muscle in 0.2*M* phosphate buffer, pH 7.5, were prepared similarly to the procedure mentioned above. The succinic dehydrogenase (SDH) activity was determined according to the procedure described by Beatty *et al.* (1966). The Biuret method was also used on the aliquots of these extracts. SDH units were expressed as mg formazan formed per mg tissue protein.

Statistical analyses

The data for the 2 × 2 factorial treatments were subjected to analysis of variance (Program BMD 02V) using the IBM 7094 computer. Correlation analyses (Program BMD 02D) were also conducted.

RESULTS AND DISCUSSION

Data on live weight gain indicated that low humidity (17–23% r.h.) or fluctuating temperatures (32 and 21°C) were detrimental to the growth of the pigs (Table 1) and were therefore environmental factors that imposed greater stress than moderate humidity (38–42% r.h.) or constant temperature (27°C).

The environmental temperatures influenced body temperatures and respiration rates. Wide fluctuations of respiration rates and body temperatures corresponded well with ambient temperatures in the fluctuating-temperature groups (H_1T_2 and H_2T_2).

Figs. 1 and 2 illustrate the rates of post-mortem pH decline and the light reflectance respectively of muscle from the four treatment groups. Faster pH decline ($P < .01$) and higher reflectance values ($P < .05$) were observed in group H_1T_2 as compared to H_1T_1; at low humidity the effects of constant versus alternating temperatures on these properties were not expressed but both groups reared in low humidity (H_2T_1 and H_2T_2)

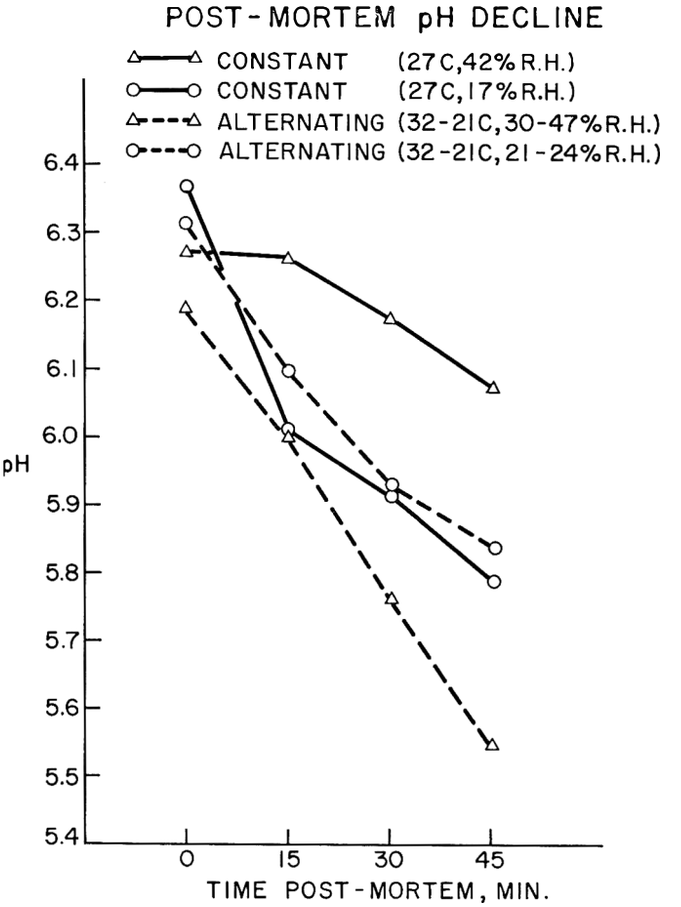

Fig. 1. Rates of post-mortem pH decline of porcine longissimus dorsi muscle from pigs reared in constant and alternating temperatures at two humidity levels.

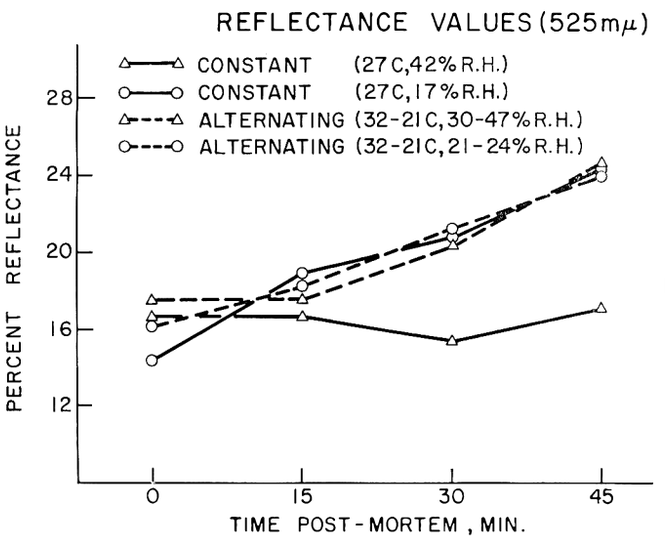

Fig. 2. Post-mortem changes in light reflectance by porcine longissimus dorsi muscle from pigs reared in constant and alternating temperatures at two humidity levels.

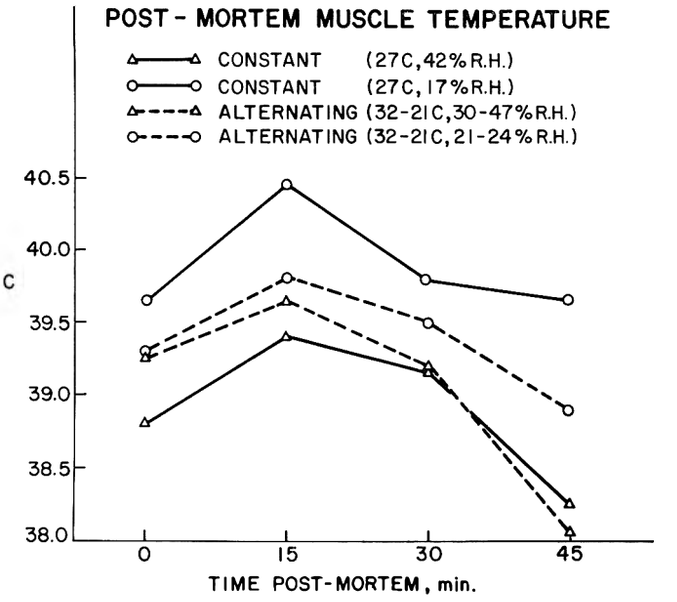

Fig. 3. Post-mortem changes in temperature of porcine longissimus dorsi muscle from pigs reared in constant and alternating temperatures at two humidity levels.

had rates of pH decline and light reflectance values of typical PSE muscle (Briskey, 1964; Figs. 1 and 2; Table 2). These results show that the extent of temperature acclimation correlated with the rate of pH decline and the color of the muscles but the interaction of temperature with humidity caused the effects to be masked when the humidity was low.

The low humidity environment resulted in higher ($P < .01$) post-mortem muscle temperatures than the moderate humidity environment (Fig. 3). Sybesma *et al.* (1966) found good correlations ($P < .01$) between pre-slaughter body temperature and post-mortem muscle temperature. These authors also showed that environmental conditions were contributory to muscle temperature and that stress (crowding, improper ventilation) induced a higher muscle temperature. These relationships and other evidences in the present study (i.e. weight gain) imply that the stress due to low humidity is as great or greater than that of the fluctuating temperature.

Individual muscles of the pig may be classified as light or dark, depending on their relative distribution of light and dark muscle fibers. Light muscles consist principally of large, pale fibers with high glycogen concentration (Beatty *et al.*, 1963) and high glycolytic enzyme activities (Ogata, 1960). Dark muscles contain small, dark fibers that have high oxidative enzyme activities (Lawrie, 1966). According to Beecher *et al.*, (1965) both longissimus dorsi and gluteus medius muscles belong to the light muscle group containing less than 30% dark fibers (based on Sudan Black B staining procedures of Ogata, 1958). In this experiment, results were expressed as light to dark fiber ratios (Table 3). In the longissimus dorsi, a significantly higher ($P < .05$) ratio was found to result from fluctuating temperature as compared to constant temperature when the humidity was moderate. It is interesting to note that correlations between light to dark fiber ratios and pH or light reflectance were high ($-.60$, $+.64$ respectively; $P < .05$) in the constant-temperature groups and low ($+0.07$, $-.02$; $P < .05$) in the alternating-

Table 2. Values of F[1] for post-mortem changes in porcine longissimus dorsi muscle as influenced by environmental conditions.

	Time post-mortem (min)			
	0[2]	15	30	45
		pH		
Humidity[3]	3.4	<1	<1	<1
Temperature[4]	1.3	1.6	2.0	3.2
H × T	<1	2.1	3.4	7.7**
		Light reflectance		
Humidity[3]	6.1*	1.5	5.3*	3.2
Temperature[4]	3.6	<1	2.9	2.5
H × T	<1	<1	3.2	4.3*
		Temperature		
Humidity[3]	4.1	7.0*	3.5	12.8**
Temperature[4]	<1	1.7	<1	3.1
H × T	3.3	3.8	<1	<1

[1] $F_{.05-1,36} = 4.11$; $F_{.01-1,36} = 7.40$ (See Figs. 1, 2 and 3 for mean values).
[2] Sample taken within 5 min of beginning of exsanguination.
[3] Ten vs 5 g of moisture/cu m of air.
[4] Constant (27°C) vs fluctuating (32, 21°C) temperatures.
* Statistically significant at the 5% level.
** statistically significant at the 1% level.

Table 3. Fiber type and enzyme activities of muscles from pigs reared in psychrometric chambers.

	Longissimus dorsi			Gluteus medius	
Group	Light:dark fiber ratio[1]	LDH[2]	SDH[3]	Light:dark fiber ratio	SDH[3]
H_1T_1	4.2	121.5	.....	2.7	.....
H_1T_2	5.4	102.5	.....	3.3	.....
H_2T_1	4.5	.....	0.43	2.9	1.42
H_2T_2	4.5	.....	0.62	2.7	0.85

[1] H_1T_1 significantly different from H_1T_2 ($P < .05$).
[2] Units/mg protein.
[3] μg formazan/mg protein.

temperature groups (combined humidity). These relationships are consistent with those reported by Addis *et al.*, (1967b) between heat stress responses and muscle properties.

Enzyme assays indicated similar activities for LDH (H_1T_1 vs H_1T_2) and for SDH (H_2T_1 vs H_2T_2) enzymes. Data were not available to compare the effect of humidity level on enzyme activity, and the environmental temperature comparisons are inconclusive. However the finding of similar LDH enzyme activities in groups of muscles (longissimus dorsi) that differed in light to dark fiber ratio suggests a need for additional investigation of the relation of fiber number and size to metabolic capabilities. It is possible that the conditions of the rearing environment may exert influences on the differentiation of skeletal muscle fibers which are accompanied by other influences on fiber mass.

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G. R. SCHMIDT, R. G. CASSENS and E. J. BRISKEY
Meat and Animal Science Department
University of Wisconsin, Madison, Wisconsin 53706

Development of an Isotonic-Isometric Rigorometer

SUMMARY—A multi-unit rigorometer has been developed to (a) make it possible to study extensibility (isotonic) and tension (isometric) changes in several muscles (red and white) simultaneously, under identical conditions, (b) make it possible to control the environment (aqueous or gaseous) around each of several muscle strips being monitored simultaneously and (c) facilitate additional biochemical studies of isotonic and isometric parameters of fresh and/or glycerinated fibers. The rigorometer is an enclosed, temperature controlled, environmental chamber designed specifically for studies on strips of parallel muscle fibers. The chamber will accommodate six muscle strips of varying sizes and has a wide adjustment for degree of sensitivity. Small cylinders have also been specially designed to facilitate separate controls of aqueous and gaseous environments surrounding each muscle strip. This multi-unit rigorometer is a further development of the rigorometer originally described by Briskey *et al.* (1962). The details of the design and application of this rigorometer are discussed.

INTRODUCTION

THE ONSET AND COMPLETION of rigor mortis is a fundamental feature of the complex changes that occur in muscle post-mortem (Bate-Smith, 1939). Historically, the development of rigor mortis has been estimated by observing changes in the extensibility of muscle strips (Bate-Smith *et al.*, 1949). The period of high and constant extensibility has been termed the "delay phase," whereas the period of diminution in extensibility has been termed the "onset phase."

The time course of changes in extensibility has been studied in muscle of the rabbit (Bate-Smith, 1939; Bate-Smith *et al.*, 1949), horse (Lawrie, 1953), whale (Marsh, 1952), chicken, (de Fremery *et al.*, 1960), ox (Marsh, 1954; Cassens *et al.*, 1967) and pig (Briskey *et al.*, 1962). However, all of the instruments previously described lack flexibility in number of samples that can be viewed simultaneously and are further limited to isotonic measurements.

Although the rigor mortis-associated shortening or tension has received some attention (Jungk *et al.*, 1967; Busch *et al.*, 1967) it has not been studied simultaneously with measurements of extensibility. The multi-unit rigorometer described in this report is a further development of the rigorometer originally described by Briskey *et al.* (1962). This unit was developed by us and assembled patent-free by E & M Instrument Co., Houston, Texas. The rigorometer described in this manuscript was designed to (a) make it possible to study extensibility (isotonic) and tension (isometric) changes in several muscles (red and white) simultaneously, under identical conditions (b) make it possible to control the environment (aqueous or gaseous) around each of several muscle strips being monitored simultaneously and (c) facilitate additional biochemical studies of isotonic and isometric parameters of fresh and/or glycerinated fibers.

DESCRIPTION OF APPARATUS

THE RIGOROMETER IS AN ENCLOSED, temperature controlled, environment chamber designed specifically for studies on strips of parallel muscle fibers (Fig. 1). The chamber will accommodate 6 muscle strips, up to 8 in. in length, with individual lever assemblies for myographic measurement (Fig. 2a and b). Isotonic myographs (Fig. 1 and 2) may be attached internally to the lever system and can be adjusted for the degree of sensitivity required, while the isometric myograph (Fig. 1) may be mounted externally to the chamber and attached to the muscle with a fine cable through a specially designed hole. Counter-balancing weights may be positioned along the lever assembly as required to balance the weight of the muscle strip.

The muscle strips are held at the bottom by an adjustable rod-and-clamp assembly, which can fix the position of the strip (a) for retention in the temperature-controlled environmental chamber, (b) for immersion in fluid in the specially designed cylinders or (c) for holding in a specific liquid and in a particular gaseous environment (Figs. 1 and 3). The clear plastic panel ends and front of the chamber are magnetically held in place and easily removable permitting ready access to any of the muscle strips as well as observation of the chamber from three sides.

An automatic load lifter (seen in Figs 1 and 2b) insures simultaneous raising and lowering of individual weights, the cycle for which can be controlled with the cycle selector (Fig. 2a).

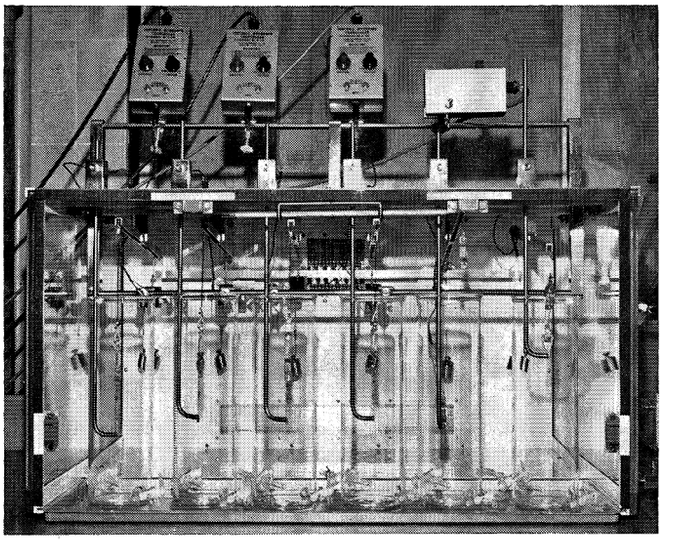

Fig. 1. Details of chamber showing positions of muscle strip and isometric transducer.

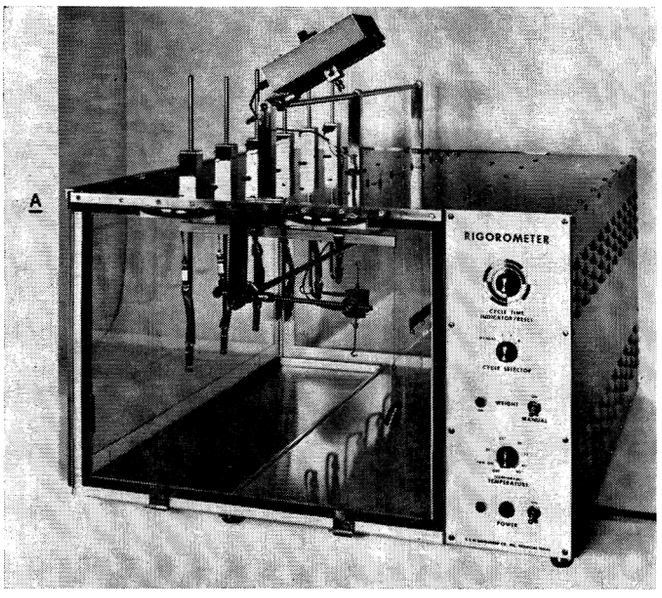

Fig. 2. End of chamber showing complete assembly for one muscle strip. A: Window and panel assembly. B: Diagram of components.

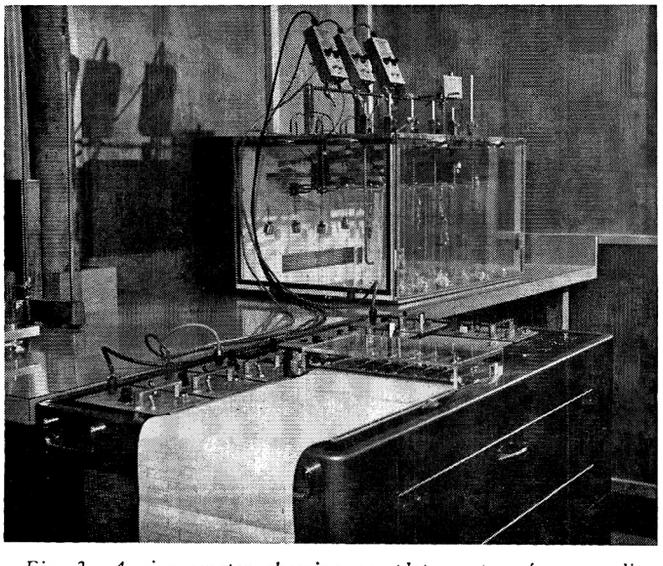

Fig. 3. A rigorometer showing complete system for recording isotonic and isometric changes.

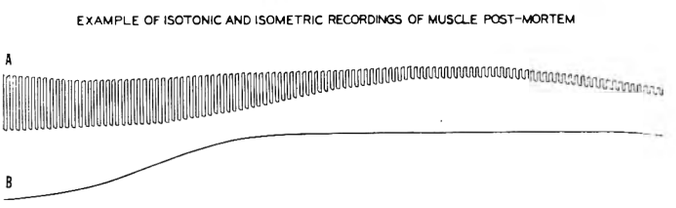

Fig. 4. Example of isotonic (A) and isometric (B) recordings of muscle post-mortem.

Fig. 3 shows the rigorometer in position for operation with a Physiograph 6, E & M Instrument Recorder. In this kind of operation the isotonic and isometric patterns may be recorded simultaneously on paper or projected onto a screen for general observation. Fig. 4 shows the typical kind of isotonic and isometric record obtainable with the equipment. The multi-unit feature of this rigorometer will facilitate time course studies of changes in the chemical constituents of the muscle strips while undergoing isometric and isotonic alterations. Chart speeds and amplification are variable. Fig. 5 shows a comparison of three widely-differing time courses of changes in extensibility.

Since the purpose of this manuscript is to describe the design and development of the multi-unit, isotonic, isometric rigorometer, it does not warrant any extensive discussion. Instead the authors wish to emphasize the flexibilities of this equipment for intensive research and/or classroom demonstration.

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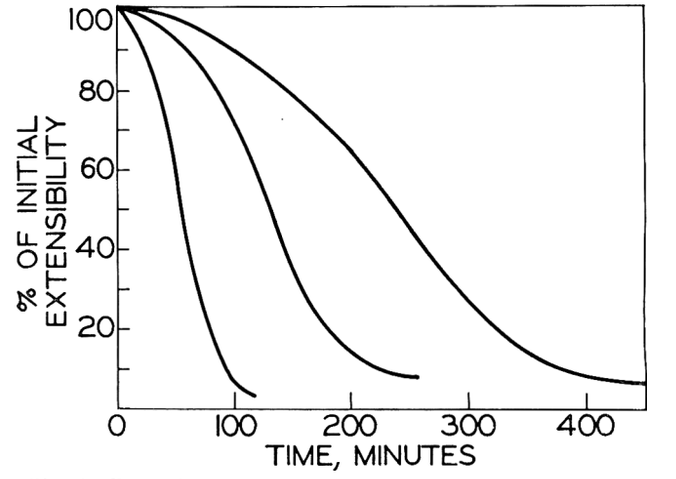

Fig. 5. Examples of graphically expressed changes in extensibility of muscles (L. dorsi) from different animals.

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F. MARTINEZ[a] **and T. P. LABUZA**

Department of Nutrition and Food Science, Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Rate of Deterioration of Freeze-Dried Salmon as a Function of Relative Humidity

SUMMARY—The rates of several deteriorative reactions, including lipid oxidation, astacene pigment loss, carbon dioxide production, and production of non-enzymatic browning pigments, were studied in freeze-dried salmon at 37°C and at several relative humidities. Results previously obtained in cellulosic model systems containing methyl linoleate were confirmed by data on oxygen absorption as a function of moisture content. Both the rate of the initial peroxide monomolecular decomposition and the peroxide value decreased as the water content was increased. Astacene pigment loss was reduced significantly by the higher moisture contents; non-enzymatic browning was increased. The significance of the reactivity of water at low moisture contents was demonstrated by its effect on the various reactions.

INTRODUCTION

FREEZE-DRYING HAS BECOME an important process for the preservation of foods because the quality of the rehydrated product is better than that of products prepared by conventional dehydration techniques. However, regardless of the success of the process itself, more investigation of the effect of the final moisture content on changes in the quality of the product during storage is necessary. The quality aspect involves reactions occurring under different conditions of time and storage, such as protein denaturation, changes in pigments, browning reactions, microbial growth, and development of oxidative rancidity. This last reaction, lipid oxidation, is one of the most important because of the highly porous nature of the product, making the lipid more accessible to oxygen, and because of the low moisture content, which tends to promote oxidation.

Several studies have been carried out to determine the factors affecting the above reactions. The results of storage tests on food items, individually and in combinations, were consistent with the idea that the amount of water adsorbed on those systems has a very significant effect (Lea, 1958; Notter *et al.*, 1959; Karel *et al.*, 1964). It has been postulated that the calculated monolayer value of moisture adsorption defines the region of highest stability for freeze-dried food items (Salwin, 1962).

The importance of the monolayer value for model systems undergoing lipid oxidation has been studied by Maloney *et al.* (1966) and Labuza *et al.* (1966). In their work it was found that the water exhibited two effects: hydration of some metals that catalyze lipid oxidation, rendering them inactive; and formation of hydrogen bonds with hydroperoxides produced to cause an extension of the monomolecular rate period.

In most studies of food materials, however, the type and amount of data taken cannot allow for an analysis of the true mechanism of the effect of water on the stability of the product. The present work was set up to determine the effect of moisture content on the rates of several deteriorative reactions in freeze-dried salmon during storage at 37°C. Oxidative rancidity, browning discoloration, and changes in pigments were studied at several moisture contents corresponding to nearly dry, below monolayer, and above monolayer coverage of water values. An analysis of the data was made to ascertain the true mechanism of the effect of water on the stability of the product in view of the work done in model systems.

MATERIALS AND METHODS

Preparation of samples

The freshest Pacific sockeye salmon *(Onocorhynchus nerka)* available was obtained from a wholesale dealer. It was frozen at −40°C, sliced into ¼-in. slabs, and freeze-dried for 60 hr at 60–100 μ Hg and room temperature in a Vacudyne pilot freeze-dryer. The vacuum was broken with pre-purified nitrogen. To eliminate the in-

[a] Present address: Universidad Agraria, La Molina, Lima, Peru.

herent variability of the material itself, the following treatment was adopted: 1) Only slices from the middle third of the fish body were used. 2) The skin and dark muscle were removed from the slices after freeze-drying. 3) The muscle near the abdominal cavity was also removed after freeze-drying. 4) The blood remaining in the tissue located adjacent to the backbone was removed.

After freeze-drying, the flesh was mixed thoroughly, and the necessary amounts were weighed into reaction flasks. Specific flasks and amounts were used for manometric studies, lipid extraction, peroxide value, pigment determination, and head-space analysis.

Moisture sorption isotherm. Using the desiccator method (Stitt, 1958), a sorption isotherm was prepared at 37°C. The plot of water activity versus moisture content is presented in Fig. 1. The value at which monolayer coverage of water occurs was calculated from the BET equation (Adamson, 1960). The monolayer was found to occur at a water activity of a = 0.19 and at a moisture content of 5% on a dry basis.

Moisture equilibration. The freeze-dried samples were equilibrated at relative humidities corresponding to moisture contents above and below the water monolayer value. Samples were placed into desiccators containing saturated salt solutions at < 0.1, 11, 32, and 40% RH, equilibrated under vacuum for 4 hr at 37°C, removed by breaking the vacuum with air at the proper relative humidity, and kept at 37°C for the subsequent study.

Moisture content determinations. To ensure complete equilibration, duplicate samples from each desiccator were used for moisture determination. This determination was made by measuring the equilibrium vapor pressure in the manner indicated by Stitt (1958), using the highly sensitive manometric system of Karel *et al.* (1964).

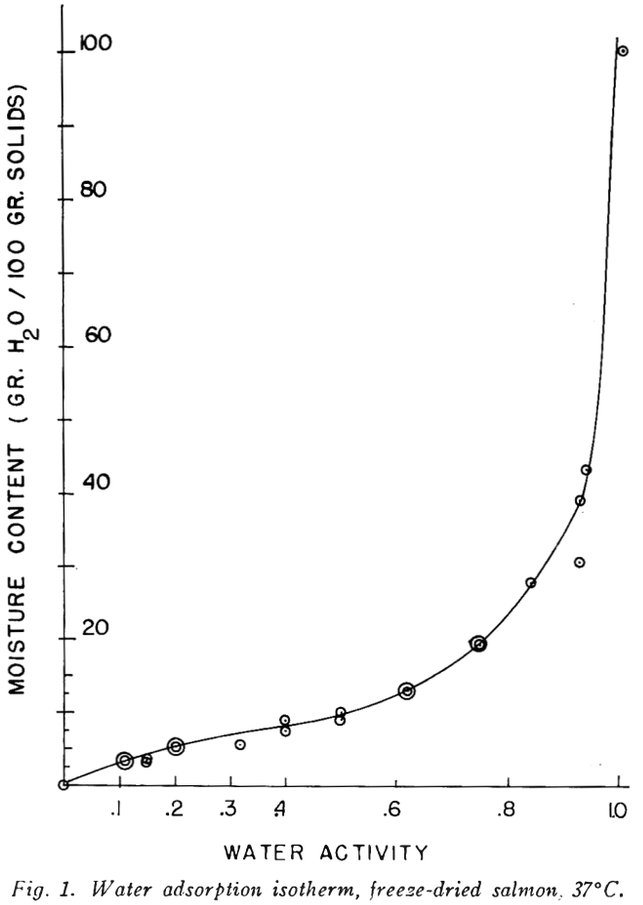

Fig. 1. Water adsorption isotherm, freeze-dried salmon, 37°C.

Stability studies

Oxygen absorption. The amount of oxygen absorbed by the freeze-dried fish was followed by the standard Warburg technique, using triplicate monometers and a thermobarometer for each relative humidity sample. Standard manometric procedures were used for calibration of the equipment and calculation of results (Umbreit *et al.*, 1964). The amount of sample was selected in such a way as to maintain a 1:25 ratio of sample volume to head-space volume. Results were expressed as microliters oxygen per gram lipid.

Correction for the carbon dioxide produced in the system was made by assuming that the volume of carbon dioxide produced is equal to the absorbed oxygen not detected by the manometers. Data from head-space analysis were used to obtain a correction for the volume of carbon dioxide evolved.

Lipid extraction. Triplicate samples were extracted with 60 cc of a chloroform-methanol (3:1) mixture, shaken for 1 hr on a rotary shaker, then filtered through a Buchner funnel. The solvent was removed on a rotary evaporator (50°C, 30 in Hg, 1 hr) and the remaining lipid weighed. The average extraction value was used for all subsequent calculations. In each case the lipid content was approximately 20% of the dry solids' weight.

Peroxide value. Results were obtained in milliequivalents oxygen per kilogram lipid by using the AOCS method on lipid extracted from fish as above but under nitrogen. In order to establish comparisons with oxygen absorption data, these units were converted to microliters oxygen per gram lipid by multiplying by 11.2 (the conversion factor).

Head-space analysis. Head-space analysis was used as an indication of oxygen absorbed and carbon dioxide evolved. Two grams of fish were put into each 50-cc Erlenmeyer flask, which was then stoppered with a rubber serum cap. Using the procedure described by Karel *et al.* (1963), 1 cc of the head space was injected during storage into an Aerograph Model A-90 P Gas Chromatograph with a thermal conductivity cell.

Peak heights were compared with standards of known gas concentration, the results being expressed as percent of the head space.

Production of browning pigments. Pigments were measured according to the method presented below (modified from Choi *et al.*, 1949).

Two g of dry material were dispersed in distilled water (20 cc). A trypsin suspension of 2.5 ml, prepared a few minutes before the analysis, was added. After a 1-hr incubation at 45°C, 2 ml of 50% trichloroacetic acid and 0.1 g Celite (analytical filter aid) were added. After mixing and filtering, the optical density of the filtrate was measured at 420 mμ, setting the enzyme blank at 100% T. The readings were made in a Beckman B Spectrophotometer.

Astacene pigment loss. The pigment was extracted by the method developed by Bligh *et al.* (1959). The optical

density of the chloroform extract of the pigment was measured at 475 mμ in a Beckman B Spectrophotometer. A reading at 390 mμ was used as a reference point to eliminate differences in the absolute amount of pigment extracted (Lusk *et al.*, 1964). The data are reported as the ratio of the optical densities at 475 and 390 mμ (OD 475/OD 390).

Fatty acid analysis

In order to make comparisons with runs in which a model system containing methyl linoleate was used and to study certain aspects of reaction kinetics on the basis of methyl linoleate alone, the fatty acid composition of the freeze-dried material was determined.

Triplicate samples were methylated according to the trans-esterification procedure (no. 1) (Jamieson *et al.*, 1965). The methyl esters were dissolved in hexane and then examined on a F & M Model 1609 gas chromatograph.

Comparison of the retention times of the fatty acid methyl esters obtained from the fish lipids with the retention times of fatty acid methyl esters of known concentration obtained from the Hormel Institute and from Applied Science Laboratories, Inc., led to identification. Conventional procedures were used for peak area measurements.

RESULTS AND DISCUSSION

THREE EXPERIMENTS VARYING from 250–900 hr were carried out on the freeze-dried salmon. As many treatments as possible were considered in each test because of the inherent variability of the fish (Lovern, 1942; Notevarp, 1961). Although the absolute magnitudes of values varied between the runs in each case, the trend observed was the same. Results are presented from typical values observed.

Oxidative deterioration

Oxidative deterioration was evaluated by measurement of the oxidation of the lipids, the protein fraction, and the destruction of astacene, the pigment responsible for the typical red color of fresh salmon. Fig. 2 shows the data, corrected for the amount of carbon dioxide produced by the sample, for the Warburg oxygen absorption of salmon. It can be seen that the major effect of humidification is to significantly decrease the over-all rate of oxidation after an initial fast phase. The protective effect of water is evident both below the monolayer (11% RH) and above it in all cases. These results are similar to those found by Maloney *et al.* (1966) for cellulosic model systems.

As was observed by Tappel (1956) and by Karel *et al.* (1966), oxygen can also be absorbed by proteins. This can account for up to 50–80% of the total oxygen absorbed, especially at high temperatures. To test for such absorption in the salmon system, in each run samples that had been extracted 6 times with $CHCl_3$:MeOH (3:1) were also placed in Warburg manometers. Very little absorption was observed. In all cases it was less than 1% of that found for the unextracted fish, so that protein oxidation was assumed to be negligible.

Oxygen absorption was also determined by head-space analysis. It was generally found that less oxygen was absorbed with increasing % RH, although the data show large deviations from the Warburg data. These discrepancies could be due to inhomogeneity of the sample since a different sample was used for each point. Similar difficulties have been reported by Vilece *et al.* (1955) and by Karel *et al.* (1966).

The results of peroxide value analyses are presented in Fig. 3. The peroxide values varied inversely with the amount of water present in the system, showing the protective effect of water.

Comparison of the peroxide value, in microliters of oxygen per gram of lipid, with the Warburg oxygen absorption data shows that the peroxide value gives a very poor indication of the actual total amount of oxygen absorbed by stored foods. This is the opposite of results found in a cellulosic model system containing methyl linoleate. This model system was prepared in the manner described by Maloney *et al.* (1966).

Oxygen absorption and peroxide value analyses were performed by following the methods described in this paper; the results are plotted in Fig. 4 and show that the correlation between peroxide value and oxygen absorbed is very good. Lundberg *et al.* (1947) studied the bulk oxidation of methyl linoleate at various temperatures and found a direct correlation between these parameters at temperatures near 40°C.

The poor correlation of peroxide value with oxygen absorbed in foods could be due to rapid breakdown of the

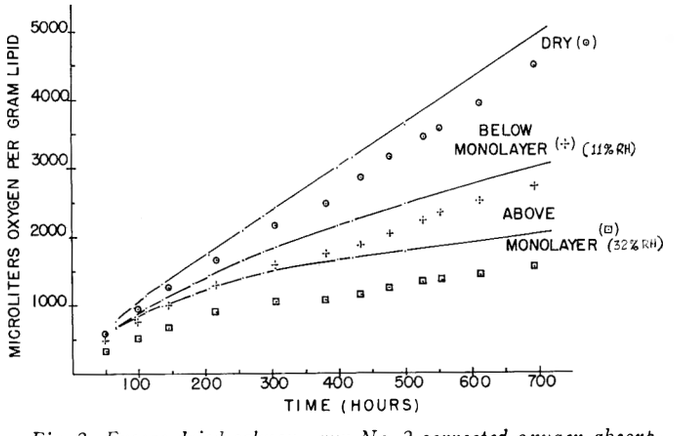

Fig. 2. Freeze-dried salmon—run No. 2 corrected oxygen absorption. (Lines above points are data corrected for Co_2 evolution.)

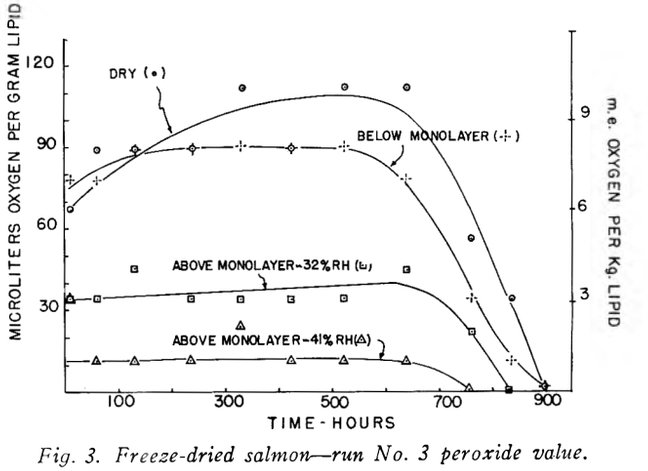

Fig. 3. Freeze-dried salmon—run No. 3 peroxide value.

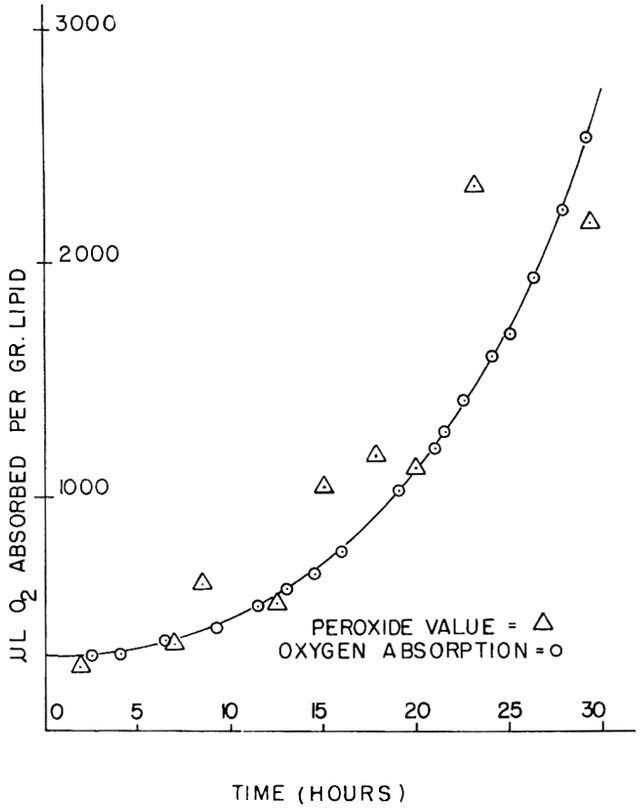

Fig. 4. Relationship oxygen absorption peroxide value in a freeze-dried model system containing methyl linoleate.

hydroperoxides due to secondary reactions, significant oxygen absorption by components other than lipid (Tappel, 1956), or irreversible chemical bonding of the peroxides to other components such as proteins, as found by Tappel (1955) and by El-Gharbawi *et al.* (1965).

It can be seen from Fig. 3 that even though the peroxide value does not show the total quantitative oxygen absorbed, the protective effect of water is readily observed if enough determinations are made during the storage period.

The effect of moisture content on the oxidative deterioration of astacene is presented in Fig. 5 for run 3. The effect is similar to that found for lipid oxidation, with values above the monolayer giving almost complete protection. Lusk *et al.* (1964) discussed the sensitivity of the

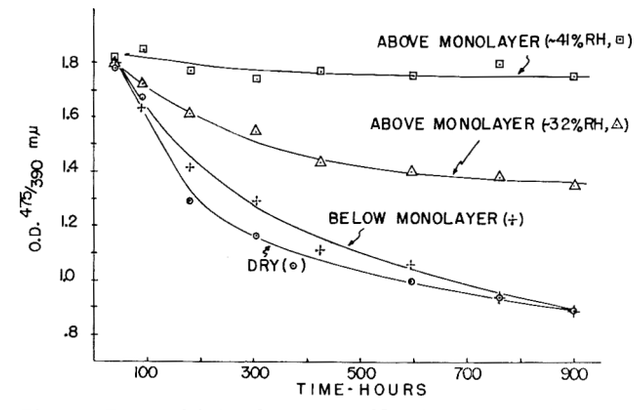

Fig. 5. Freeze-dried salmon—run No. 3, astacene pigment deterioration.

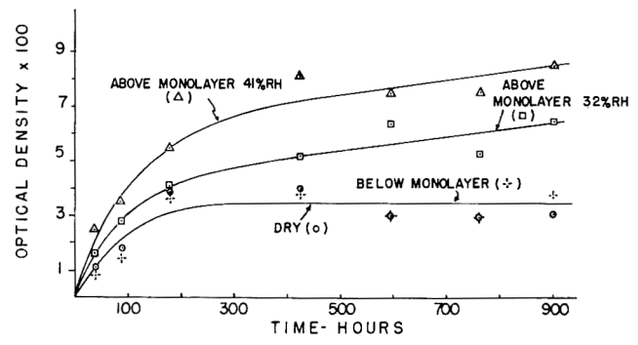

Fig. 6. Freeze-dried salmon—run No. 3, browning pigments production.

pigment when exposed even to low oxygen partial pressure, with stability obtained only in pure nitrogen at 0°F. The present results show that complete stability can be obtained at 37°C in air by choosing the proper moisture content.

Non-enzymatic browning

The extent of non-enzymatic browning deterioration was evaluated by determining the relative amounts of pigments and carbon dioxide produced during the course of the reaction. Because the data were obtained from analyses performed on different samples and homogeneity may not have been attained during their preparation, the variability of the results is large.

Production of brown pigments. The results of determinations performed on aqueous extracts of similar quantities of freeze-dried material are presented as OD × 100 versus time in Fig. 6 and 7 for 2-g samples. It can be seen that the rate of production of pigments was a function of the amount of water in the material studied, except in the case of the dry sample (run 2, Fig. 6) where a secondary reaction presumably complicated the mechanism. It is possible that 2 types of browning took place: one in which the residual free reducing sugars present in the freeze-dehydrated product participated in reactions with the amino groups of the protein and one in which the decomposition products of oxidized lipid probably reacted with the amino groups of the protein.

According to Jones (1962) glucose, ribose, and phos-

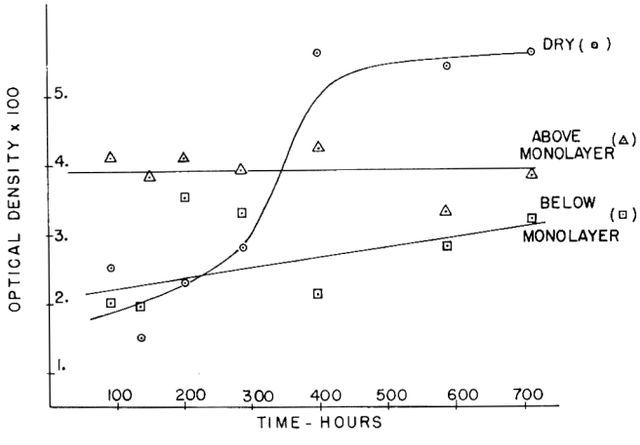

Fig. 7. Freeze-dried salmon—run No. 2, browning pigments production.

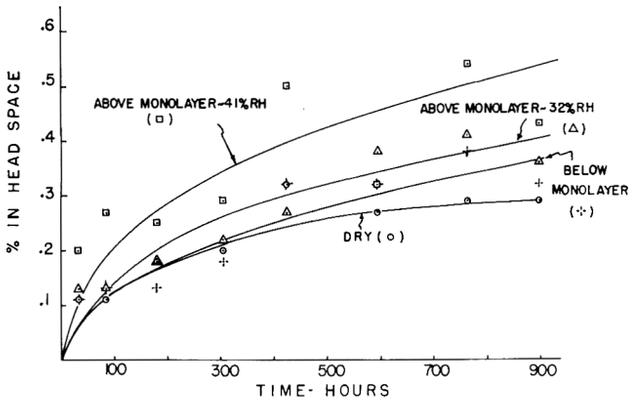

Fig. 8. Freeze-dried salmon—run No. 3, carbon dioxide production.

phorylated hexoses are the most important compounds responsible for sugar-amino reactions in dried fish products. The significance of the relative amounts of free reducing sugars varies, depending upon many factors including physiological conditions and processing variables. These have been studied extensively by Tarr *et al.* (1965), who have shown that moisture definitely controls the rate of browning.

The curves obtained for the samples equilibrated at 11, 32, and 41% RH in run 3 are characteristic of a non-enzymatic browning process taking place in a system where the concentration of reducing sugars has become limiting after a certain reaction time. Also, it is clear that the rates are proportional to the amount of water available for the transport of the reactants in solution. Since the rate of oxidation of the samples was very slow at this time, the observed browning probably occurred via an interaction of free reducing sugars and amino groups of the protein.

The faster rate of browning exhibited by the dry samples (Fig. 6) can be explained on the basis of the reaction of protein end groups with products of oxidation (Lea, 1958; Toyomizu *et al.*, 1963). These decomposition products of oxidized lipids, probably aldehydes and ketones, may be responsible for the initiation of non-enzymatic browning reactions at levels of moisture near dryness (Tarr *et al.*, 1965).

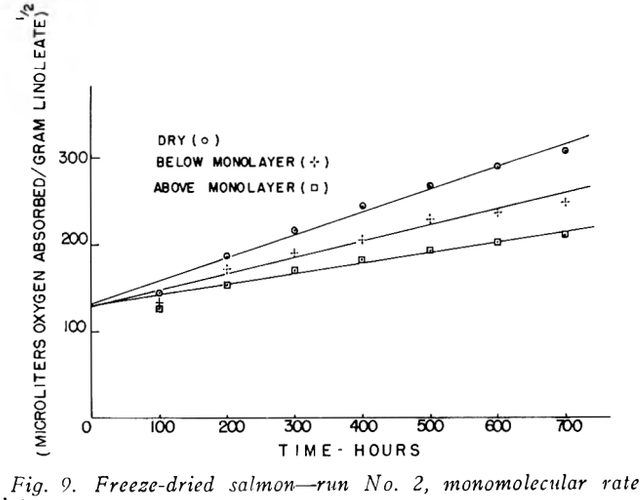

Fig. 9. Freeze-dried salmon—run No. 2, monomolecular rate plot.

Results of GLC head-space determinations are presented in Fig. 8 as percent carbon dioxide in head space versus time. The data represented in this figure show that the evolution of carbon dioxide paralleled the production of pigments, indicating that carbon dioxide is produced during browning and is probably produced through Strecker degradation.

Kinetics of deterioration

Effect of humidification on the rate of lipid oxidation of freeze-dried salmon. Examination of the oxygen absorption curves in Fig. 2 indicates that one of the effects of water is a reduction of the relative amounts of oxygen absorbed. To elucidate the actual mechanism for this protective effect, the results were recalculated on the basis of oxidizable lipid. It has been demonstrated (Lundberg, 1962) that the rate of oxidation of fatty acids containing less than 18 carbon atoms and less than 2 double bonds is negligible compared to that of linoleic acid.

The results of the fatty acid analysis performed on the freeze-dried salmon in this study showed 4.8% linoleic acid and 0.17% linolenic acid. Saddler *et al.* (1966) found 11.7% linoleic acid and 1% linolenic acid while the USDA (1963) reported only 1.4% linoleic acid. These differences could be due to the various factors mentioned previously. It is reasonable to assume that linoleic acid is the main fatty acid responsible for the observed oxygen uptake. The other fatty acids found were either non-oxidizable or were found only in trace amounts.

If every oxygen molecule reacts with 1 molecule of linoleic acid to yield a linoleic acid hydroperoxide, and if the concentration of linoleic acid is large enough to be considered constant throughout the oxidative period, then the plot of the square root of the oxygen absorbed per gram of linoleic acid versus time will be a straight line whose slope is a measurement of the monomolecular rate constant (Maloney *et al.*, 1966). A monomolecular rate plot for run 2 is presented in Fig. 9.

With the model system previously discussed, the reaction proceeds by a monomolecular decomposition of the hydroperoxides until a value corresponding to approximately 25 (μl O_2 absorbed/g lipid)$^{1/2}$ is reached; the change in slope obtained afterwards indicates that the biomolecular phase of oxidation is taking place with the breakpoint increasing with moisture content (Karel *et al.*, 1967). The data obtained for the salmon demonstrate the following 3 points.

Since no upward breakpoint exists, the oxidation of the lipids of freeze-dried salmon does not enter into the rapid bimolecular decomposition phase. This result is reasonable since, in complex materials such as foods, peroxides formed have less opportunity to reach each other and to decompose bimolecularly.

The data do not fit into a straight line very well. The reason for this is that the amount of oxidizable lipid (linoleic) may not be constant throughout the observation period; however, it could become limiting after a certain reaction time, thus causing the monomolecular rate plot to curve downward. The results are based on a lipid composition taken from a single fish; because of reasons

mentioned previously, the true value may vary considerably.

Since some browning occurred with increased moisture content in the salmon, end products of browning may be responsible for the decrease in rate through an anti-oxidant mechanism (see review of mechanism by Lea, 1958).

Monomolecular rate constants obtained for the salmon and for the model system are compared in Table 1, which shows that the effect of moisture was exerted by a lowering of the monomolecular rate constants. This indicates the possible occurrence of hydrogen bonding of hydroperoxides by water, thereby preventing their entering into initiation reactions, and of inactivation of metal catalysts by water. These results are in agreement with the work of Maloney *et al.* (1966) and Labuza *et al.* (1966).

Effect of humidification on the rate of astacene deterioration. According to Lusk *et al.* (1964) the pigment astacene is extremely sensitive to oxidation even at very low oxygen pressures; therefore, if the concentration of oxygen is not limiting, a first-order mechanism can be proposed with the rate of the reaction at any instant proportional to the concentration of substrate left. Thus,

$$\ln \frac{A}{Ao} = -K\ominus$$

where Ao is the initial amount of pigment;

A is the amount present at time $\ominus$;

K is the first-order constant for the reactions; and

$\ominus$ is time.

The same analysis has been applied by Falconer *et al.* (1964) in a study of the oxidation of carotene in dehydrated carrot.

The first-order rate plot for the salmon is presented in Fig. 10, and the numerical values for the constants obtained as a function of different humidification treatments are presented in Table 2. From these data it is possible to conclude the following:

Water exerted a marked effect by lowering the rate of deterioration of astacene.

The protective effect of moisture was manifested continuously from the multilayer region to the dry state. The actual manner by which water protects astacene from oxidation cannot be truly elucidated. Since the data nearly fit into a straight line, demonstrating that the concentration of oxygen is not limiting, the theory proposed by Halton *et al.* (1937) and by Salwin (1962)—that water forms a protective film excluding oxygen from the surface—cannot be of significance. However, there are two mechanisms that can be operative in the system studied.

The oxidative reaction is probably affected by metal catalysis, and water hydrates metals, thus rendering them inactive. Secondly, the reaction may only be pseudo-first order over the range of concentrations studied. If the reaction obeys a free radical mechanism instead, then, as in the case of lipids, the effect of water on the oxidative deterioration would be exerted as found previously. In the present case the concentration of free radicals may be high enough to produce the pseudo-first order dependence.

Table 1. Monomolecular rate constants for freeze-dried salmon ([μl O_2/g linoleate]$^{1/2}$ per hr).

Samples	Dry < 0.1% RH	Below monolayer ~ 11% RH	Above monolayer ~ 32% RH	Above monolayer ~ 40% RH
Model system[1]	0.47	0.30	.....	.....
	0.94	.....	0.66	.....
	1.23	.....	.....	1.0
Salmon: Run 2	0.54	0.38	0.24	.....
Run 3	0.5	0.5	0.43	0.36

[1] Data taken from Karel *et al.*, 1967.

Table 2. Astacene pigment rate constant (hr^{-1}).

	Dry < 0.1% RH	Below monolayer 11% RH	Above monolayer 32% RH	Above monolayer 40% RH
Run 2	1.1×10^{-3}	0.9×10^{-3}	0.6×10^{-3}	.....
Run 3	1.1×10^{-3}	1.0×10^{-3}	0.5×10^{-3}	0.1×10^{-3}

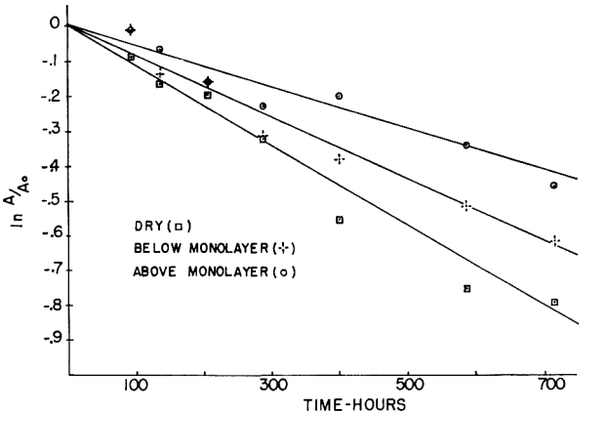

Fig. 10. Freeze-dried salmon—run No. 2, astacene deterioration—first-order plot.

Effect of humidification on the rate of non-enzymatic browning. According to Ellis (1959) the amount of pigment formed during non-enzymatic browning reactions is directly proportional to the square of time elapsed and to the concentration of reactants. Thus,

$$\text{browning pigment} = K(A)^2(S)\ominus^2$$

where A is the concentration of amino acid end groups;

S is the concentration of reducing sugars;

$\ominus$ is the reaction time; and

K is the rate constant.

In the salmon system the quantity of amino acid end groups is large, and the carbonyl compounds produced during the oxidation of lipids can accomplish the role of sugars (in addition to the free sugars present). Therefore, the equation can be reduced to

$$OD_{420} = K'(Y)\ominus^2$$

where OD_{420} is the concentration of pigment formed and Y is the total quantity of reducing substances capable of undergoing the reaction.

A plot of the concentration of pigment formed (OD_{420}) versus the square of the time elapsed will be a straight line whose slope is proportional to the rate of the reaction and to the concentration of reducing compounds available. This plot is presented in Fig. 11 for run 3. A sudden increase in the slope is attained during the period when the oxidation of the more reactive lipids probably takes place. This fact supports the observation made in this study by show-

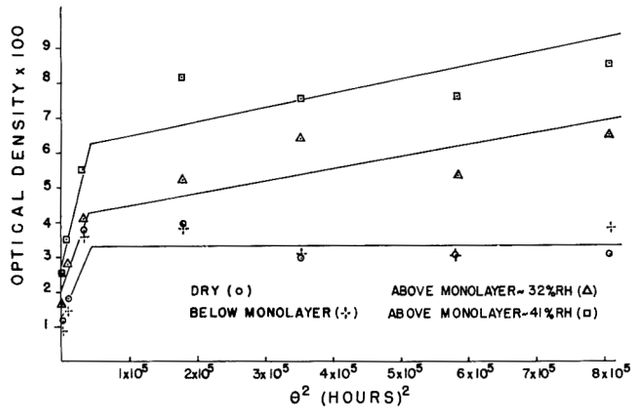

Fig. 11. Freeze-dried salmon—run No. 3, browning kinetics.

ing that products of the autoxidation reaction of lipids probably interact with the amino groups of the protein to form carbon dioxide and browning pigments.

In addition, at the end of the period where the rate of lipid oxidation was rapid the sudden rise in browning pigments stopped. This could be explained by the dual role of oxidation and browning as a function of moisture content. The water promoted browning, which produced anti-oxidants, thus causing the rapid oxidation period to cease.

CONCLUSIONS

IN THE STORAGE OF DEHYDRATED FOOD the equilibrium relative humidity of the final product will largely determine the storage life of a food. In a product such as salmon a balance must be achieved among the various deteriorative reactions in order to predict the maximum storage life. Since it has been shown that, even below the monolayer, water exerts a distinct effect, the use of the monolayer value as the optimum condition may not be desirable. In this study it was found that at 32% RH the salmon showed the best over-all stability. This is in excess of the monolayer and far in excess of the recommended 2% moisture content maximum for most dehydrated foods.

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J. C. CRIGLER and L. E. DAWSON
Department of Food Science, Michigan State University
East Lansing, Michigan 48823

Cell Disruption in Broiler Breast Muscle Related to Freezing Time

SUMMARY—Cell disruption, resulting from different freezing times, was evaluated by studying the composition and amount of drip obtained from broiler breast muscles after freezing and thawing. The degree of cell disruption was estimated after measuring the amount of drip released and by total solids, nitrogen and deoxyribonucleic acid (DNA) concentration of the drip. Initial drip release was noted approximately 5½ hr after the frozen meat was placed in a refrigerator at 16°C, and collections were made through the 18th hr. Degree of cell disruption was not uniformly related to changes in freezing times of 0.5 to 1,494 min. In general, increased freezing time resulted in greater cell disruption; however, several exceptions were noted. Cell disruption was relatively severe for tissues frozen in 18 to 35, 87, and 252 min, and relatively low for tissues frozen in times of 1 to 18 min, 132 to 225 min, and longer than 1,044 min. All frozen and thawed muscles had higher contents of total solids, nitrogen and DNA than unfrozen controls.

INTRODUCTION

POULTRY MEATS are commonly frozen to preserve quality during distribution or holding prior to sale and/or consumption. Poultry meats are prepared for freezing as whole carcasses, cut-up pieces or boneless products. There has been a definite increase in the preparation and utilization of the latter for boneless rolls, poultry pot pies and similar type products.

After poultry meat, especially that processed as cut-up or ready-to-cook, has been frozen and thawed, some fluid exudes from the products and collects as drip. Koonz *et al.* (1939a,b), Callow (1952), Empey *et al.* (1954), Love (1955a) and Wladyka (1965) reported that amount of drip released was influenced by freezing time. Disruption of cells of frozen fish were reported in detail by Love (1955a,b; 1958a,b,c). He measured the DNA in the fluid expressed from fish fillets on the assumption that it would appear only when the muscle fibers had been burst open and the amount of DNA would provide an index of cell damage. The cell damage (disruption) has been considered to be the result of either one or both of the following: (a) denaturing of cellular protein by the concentration of salts during freezing (Callow 1952, 1955), or (b) cell disruption by the formation of ice crystals (Koonz *et al.*, 1939a).

This study was conducted to evaluate the influence of freezing rate on cell disruption in poultry tissues as measured by amount and composition of drip.

MATERIALS AND METHODS

Processing of broilers

Nine-week-old broilers were slaughtered, scalded in a Rotomatic scalder and picked in a Cyclomatic rubber-fingered picker. After processing, the carcasses were washed and placed in slush ice for 8 hours. At the end of the chilling period, the carcasses were removed from the slush ice and the breast muscles (*Pectoralis major* and *minor* muscles) were carefully removed from the carcasses and each breast packed individually in a 6 × 10-in. Cryovac bag.

Michigan Agricultural Experiment Station Number 4146.

Freezing

Variation in freezing times was accomplished by use of liquid nitrogen, dry ice and acetone, freezers at different temperatures and varying amounts of insulation around the muscles. Freezing time is defined as the time elapsed during which the temperature of the meat changed from 0 to −5°C. Temperature was measured with a Honeywell-Brown automatic recording potentiometer with attached thermocouples. The thermocouples were inserted through small openings in the Cryovac bags and positioned in the center of the largest two muscles in each sample. After the samples passed through the temperature range of 0 to −5°C, they were stored for 1 to 3 weeks at −18°C until thawed for drip collection and analysis.

Drip collection

All samples were thawed at 16°C for 18 hr, and drip was collected in a saturated atmosphere using a method similar to that reported by Pearson *et al.* (1959). After the packaging material was removed from the frozen meat samples, they were suspended above a funnel by a clamp with the anterior end of the muscle down. A few drops of chloroform were put into the graduated cylinder to reduce microbial spoilage of the drip. Thawing time (time from −5 to 0°C) was 230 min, and the first drip was released after 330 min. At the end of the thawing period, the meat samples were reweighed, pH readings of drip and meat taken, and the drip measured to the nearest 0.1 ml. The drip was stored at −23°C prior to evaluation.

Drip rate was calculated after measuring the amount of drip released at the end of 540, 660, 900 and 1,080 min. Preliminary studies indicated that these particular time periods, except the last, resulted in sufficient drip for analyses.

Method of analysis

Ten ml of diluted drip (5 ml drip + 20 ml water) was pipetted into a tared evaporating dish to which an equal volume of 95% ethanol was added and dried to a constant weight in a drying oven (100 ± 2°C). Difference between wet and dry weights was reported as total solids.

All nitrogen determinations were made using the micro-Kjeldahl method as outlined by the American Instrument Company (1961).

Table 1. Effect of freezing time on rate and percentage of drip released from broiler breast meat during thawing.

Freezing times (min)	Thawing time periods (hr)				Total drip
	5½–9[1]	9–11	11–15	15–18	
	Percent of total drip released per hr[2]				Percent of frozen wt
2	11.4	14.9	5.3	3.1	4.6
291	12.0	16.9	4.4	2.3	7.3
1869	10.7	17.0	5.3	2.5	7.7
Ave.	11.4	16.3	5.0	2.6	

[1] First drip released after 5½ hr at 16°C.
[2] Duplicate samples each thawing time period and two replicate samples of four birds for each freezing treatment.

DNA

DNA was extracted from the drip samples using the procedure suggested by Ogur *et al.* (1950). A blue color was developed by the reaction of the diphenylamine reagent with deoxyribose according to the procedure of Burton (1956). Absorbency of the diphenylamine-deoxyribose mixture was read at 600 mμ on a Beckman DB Spectrophotometer and recorded. These readings were used to quantify the DNA concentration from a standard curve.

RESULTS AND DISCUSSION

Table 1 shows the percentage of the total drip collected per hr during each period from samples that had been frozen at times of 2, 291, and 1,869 min. Freezing time had little effect on the average percent drip released per hr.

Average pH values varied from 5.6 to 6.0. Although no significant correlation coefficients were found between pH and the amount of drip among treatments, the pH of the drip decreased slightly from samples collected at each successive thawing period. This decrease in pH could be due to increased mobility of organic acids at higher temperatures or to a change in the charge on the protein (Whitaker, 1959).

Cell damage was evaluated by determining the amount of drip released and the concentration of the total nitrogen, total solids, and deoxyribonucleic acid (DNA) in the drip from samples frozen at 17 different rates. Percentage drip and total solids in that drip collected from meat which had been frozen in different times are shown in Fig. 1. Meat frozen in 87, 252 and 1,042 min resulted in maximum

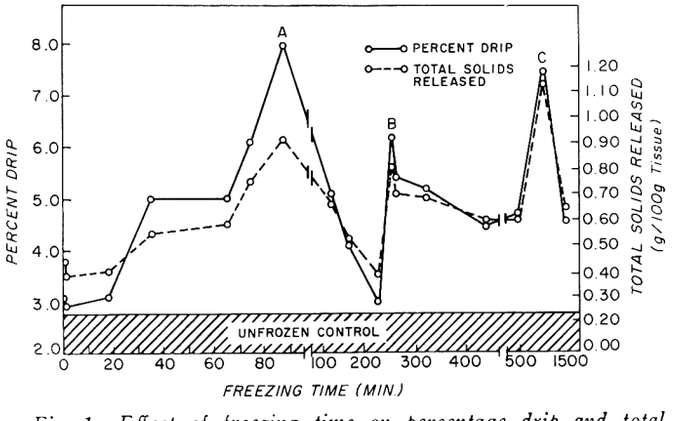

Fig. 1. Effect of freezing time on percentage drip and total solids released from chicken muscles.

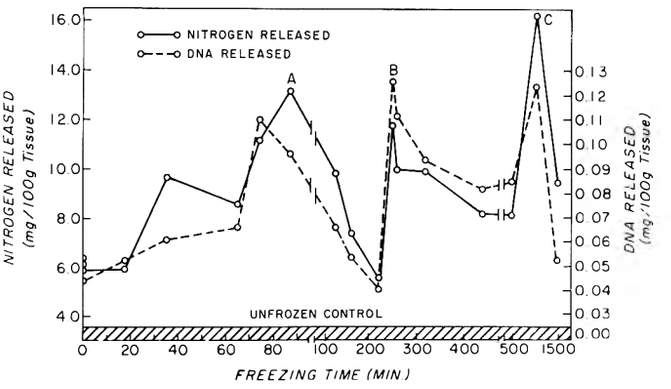

Fig. 2. Effect of freezing time on amounts of nitrogen and DNA released from chicken muscle.

drip and maximum total solids in that drip. This indicates maximum cell rupture (Ramsbottom *et al.*, 1939, and Dyer *et al.*, 1956) increased solubility in salt solution or a solvent effect by a high salt concentration without actual cell rupture (Callow, 1952, 1955). These data indicate a direct relationship between percentage of drip and total solids released per 100 g of tissue.

A direct relationship between total nitrogen and DNA in the drip was also apparent as indicated by Fig. 2. Maximum cell damage thus was indicated at three freezing times, 87, 252, and 1,042 min by drip release, total solids, total nitrogen, and DNA in the drip. These results are in essential agreement with the results reported by Love (1955a, 1958a,b) after studying frozen fish muscles.

The increased amount of nitrogen released at approximately the above three freezing times could be the result of either release of intracellular fluids which accounts for most of the nitrogen present in drip as suggested by Seagram (1958), or by the presence of cell fragments which were the result of ice particles breaking the cell walls.

A tendency for increased cell damage was shown with a freezing time of 0.5 min (Figs. 1 and 2). The results for total solids and nitrogen per ml of drip indicated that when the tissue was frozen in liquid nitrogen, the total nitrogen and total solids per ml was only exceeded in tissue frozen in times longer than 1,000 min. All frozen and thawed muscles had higher contents of total solids, nitrogen and DNA than unfrozen controls as shown by hatched area in Figs. 1 and 2.

The decrease in destruction of the tissue with freezing times in excess of those causing Peak C was believed to be the result of the fibers forming clumps with very slow freezing. Thus, the fibers in the middle of the clumps were protected from ice damage by the surrounding fibers. The larger fiber clumps, formed during increased freezing times, protected the inner fibers from damage.

The occurrence and dominance of the extensive cell damage occurring at Peak C seemed to be of considerable importance. Peak C was believed to be the result of a severe solvent effect on the cell walls by the resulting concentrated salt solutions. The fiber clumping suggested by Love (1955a) for very long freezing rates was not sufficient to offer protection of enough individual fibers, allowing considerable protein denaturation. The protein

denaturation reduces the ability of the proteins to reabsorb the fluid, increasing the amount of drip resulting in excessive leaching of the tissue materials.

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H. E. PATTEE[a] **and H. E. SWAISGOOD**[b]
North Carolina Agricultural Experiment Station, Raleigh, North Carolina 27607

Peanut Alcohol Dehydrogenase. 1. Isolation and Purification

SUMMARY—Alcohol dehydrogenase (alcohol:NAD$^+$ oxidoreductase EC 1.1.1.1) has been isolated and purified from peanut kernels. The resulting preparations exhibited a high degree of purity as shown by the criteria of ultracentrifugation and free boundary and zonal electrophoresis. The simultaneous purification of zinc and enzymatic activity indicates that peanut alcohol dehydrogenase is a zinc metalloenzyme.

INTRODUCTION

ALCOHOL DEHYDROGENASE (alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) is widespread in nature, occurring in animal tissue, plants, and microorganisms (Sund *et al.*, 1963). The enzyme has been purified and crystallized from liver (Dalziel, 1958) and yeast (Racker, 1950; Hayes *et al.*, 1954) and the enzymatic properties have been extensively investigated. However, alcohol dehydrogenase obtained from higher plants has not been highly purified or characterized.

Davison (1949) reported alcohol dehydrogenase activity to be widely distributed in the seeds of higher plants and noted that the activity changed throughout the life cycle of peas. Stafford *et al.* (1953) reported an alcohol dehydrogenase in wheat germ extracts that catalyzed the reduction of both NADP$^+$ and NAD$^+$. Suzuki (1966) reported the isolation and partial purification of alcohol dehydrogenase from pea seedlings.

Pattee *et al.* (1965) reported that ethanol is a major constituent of the volatiles from peanut kernels cured at 55°C, but not of those cured at 22°C and postulated that it was one of the major contributors to the off-flavor of high-temperature-cured peanuts. Thus development of a method of purification of alcohol dehydrogenase from peanut kernels was important so that the properties of this enzyme could be investigated and its relationship to the ethanol content of the peanut kernels evaluated.

The present communication reports a method for the isolation and purification of alcohol dehydrogenase from peanut kernels; several criteria indicate that the preparation is homogeneous.

EXPERIMENTAL

Materials

Peanut kernels (*Arachis hypogea* var. N.C. 2) were from intact peanut pods cured at 22°C to 10 percent moisture (wet weight basis) and stored at 10°C until used.

Sephadex G-150 was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey. Diethylamino-

[a] U. S. Department of Agriculture, ARS, MQRD.
[b] Department of Food Science.

ethyl (DEAE) cellulose, with 0.61 mg of basic groups per g, was obtained from Bio-Rad Laboratories. A special enzyme-grade ammonium sulfate with a low content of metal ion impurities was purchased from Mann Research Laboratories. The coenzymes NAD^+, NADH and $NADP^+$ were obtained from Calbiochem. All other chemicals were analytical grade reagents and were used without further purification.

Method of assay

Activity of the enzyme was assayed by a method similar to that described by Racker (1950). The final 3-ml volume assay mixture contained 0.1*M* ethanol, 0.5 m*M* NAD^+, and 0.01*M* sodium pyrophosphate buffer (pH 8.5), in addition to the enzyme. The reaction was initiated by adding 0.1 ml of enzyme solution to 2.9 ml of the other components. The rate of NAD^+ reduction was determined by following the production of NADH as indicated by the appearance of the characteristic absorption band at 340 mμ. Absorbance was monitored with a Beckman DU spectrophotometer [a] fitted with a Gilford Adaptor for digital readout. Readings 15 and 45 sec after the reaction was initiated were used to calculate the rate of absorbance change.

One unit of enzymatic activity was defined as the amount of enzyme resulting in the production of one μmole of NADH per min under the conditions of the assay. The molar absorbance coefficient of NADH was taken as 6.22×10^6 cm^2 per mole at 340 mμ (Horecker *et al.*, 1948).

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951), with crystalline bovine plasma albumin (Sigma Chemical Co.) as a reference standard.

Electrophoresis

Free-boundary electrophoresis was performed at ice-bath temperatures using a Perkin-Elmer Model 38A Electrophoresis Apparatus. Experiments were conducted in both 2-ml and 6-ml Tiselius-type cells. Diffusate from dialysis of the protein solution was used in the buffer compartments.

Cellulose acetate strips were used as the support medium for the zonal electrophoresis. Electrophoresis was performed in a Gelman Rapid Electrophoresis Apparatus using a current of 1 mA per strip. The strips were stained with Amido Black 10B for detection of protein, and alcohol dehydrogenase activity on the strip was determined by the procedure of McKinley-McKee *et al.* (1965). Following electrophoresis the strips were dipped into 60 ml of 0.06*M* sodium pyrophosphate buffer (pH 8.6), containing 30 μl of ethanol, 12 mg of NAD^+, 2 mg of phenazine methosulfate, and 20 mg of nitro-blue tetrazolium. The strips were incubated at 37°C in a closed box with an open beaker of the reaction mixture (to keep the strips moist) until the purple formazan bands were clearly visible.

Sedimentation experiments were performed in a Beckman Spinco Model E Analytical Ultracentrifuge. Two solutions were centrifuged simultaneously in Kel-F centerpieces with a positive wedge window in one cell permitting the schlieren diagram of each cell to be photographed on Metallographic plates.

Zinc analysis

Zinc was determined by atomic absorption spectrophotometry according to the method of Fuwa *et al.* (1964). Aliquots were taken at various stages of purification and zinc was analyzed directly with a Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer.

RESULTS

Enzyme purification

All operations were at room temperature (22°–25°C) unless otherwise specified. Peanut kernel acetone powder was prepared by homogenizing the kernels in 5 volumes of acetone with a Waring blender for 1 min at −15°C. The homogenate was filtered and the resulting powder was re-extracted 4 times each with 5 volumes of cold acetone. The powder was then extracted twice with 5 volumes of cold diethyl ether. The ether was removed by filtering through a Buchner funnel. The powder was dried thoroughly after the final ether extraction and stored at −10°C until use. When ether extraction was omitted in preliminary trials, contamination by lipid materials seriously encumbered further purification.

The acetone powder was extratced with 20 volumes of 0.1*M* sodium phosphate buffer (pH 7.5) for 1 hr at 22–25°C with continuous stirring. The mixture was centrifuged for 10 min at 12,000 × g and 0°C and the supernatant was filtered through Whatman #1 filter paper to remove floating particles. Further extractions of the residue failed to yield additional enzyme activity. The supernatant was heated rapidly to 55°C and maintained at that temperature for 15 min in a constant-temperature bath. After cooling, the mixture was centrifuged for 15 min at 35,000 × g and 0°C. To the clear supernatant 22.1 g of ammonium sulfate was added per 100 ml of solution. After standing for 30 min at 0°C the mixture was centrifuged as in the previous step and the inactive precipitate was discarded. To each 100 ml of supernatant was added an additional 14.9 g of ammonium sulfate. After standing for 1 hr at 0°C, the mixture was again centrifuged and the supernatant discarded.

The precipitate was redissolved in 10 ml of 0.1*M* phosphate buffer (pH 7.5) and 3 g of washed DEAE cellulose, suspended in 10 ml of the same buffer, was added. The resulting suspension was mixed thoroughly and allowed

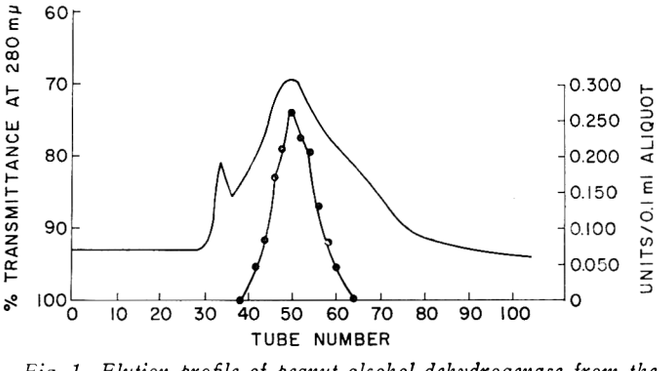

*Fig. 1. Elution profile of peanut alcohol dehydrogenase from the second Sephadex G-150 column. Column 45 × 1.5 cm; load, 65 mg of protein in 5 ml 0.1*M *phosphate buffer (pH 7.5). Eluent: 0.05*M *phosphate, pH 7.5. Rate of flow, 0.5 ml per min. Volume per tube, 2.0 ml. Temperature 22°C.*

Table 1. Typical purification chart for peanut alcohol dehydrogenase.

Fraction	Volume (ml)	Protein (mg/ml)	Total units	Sp. Act. (units/mg protein)[1]	Recovery %	Zinc g-atoms/ Mole protein
Crude extract	400	9.75	491	0.12	100	0.15
Heat treated	385	8.80	409	0.12	83	0.18
Ammonium sulfate precipitate (37–60% saturation)	125	4.63	187	0.32	38	0.58
DEAE-cellulose eluate	85	4.88	162	0.39	33	0.65
Active fraction from Sephadex G-150 (first elution precipitated from 70% $(NH_4)_2SO_4$)	21	1.33	46	1.65	9	1.50
"Peak" tube	2	1.15	5	2.17	1	

[1] A unit is defined as a micromole NADH formed per minute.

to stand for 5 min. The solution was filtered through Whatman #1 filter paper and the residue washed with 5 ml of 0.1*M* phosphate buffer, pH 7.5. The filtrate was applied to a column of G-150 Sephadex, 45 × 1.5 cm. The Sephadex was allowed to swell in water for at least 48 hr before column preparation. The columns were prepared (Andrews, 1964) and allowed to equilibrate with 0.05*M* phosphate buffer, pH 7.5, for 4 hr at 22–25°C. Using 0.5*M* phosphate as the eluting buffer, 2 ml fractions were collected and the eluent monitored at 280 mμ. The fractions containing enzyme activity were combined and the protein precipitated with ammonium sulfate, 39 g per 100 ml. The mixture was allowed to stand for 30 min and then centrifuged for 15 min at 35,000 × g and 0°C. The precipitate was dissolved in 5 ml of 0.1*M* phosphate buffer, pH 7.5, and applied to a fresh column of Sephadex G-150, eluting as before.

The profile of a typical second elution from G-150 Sephadex is shown in Fig. 1. The position of enzymatic activity is also indicated. Two protein "peaks" other than that due to active enzyme are distinctly visible. Whether these represent inactive enzyme or contaminating proteins is not known. The greatest increase in specific activity occurred as a result of the first elution from Sephadex (Table 1). The data in Table 1 also indicate an 18-fold purification was obtained when contamination from inactive protein is minimized by selecting the 2-ml fraction with the highest concentration of protein and activity from the second Sephadex column.

The zinc content at various levels of purification is also shown in Table 1. Comparison of the increasing levels of zinc with corresponding increased specific activity conclusively shows that zinc and enzymatic activity are purified simultaneously. Furthermore, the data indicate a 10-fold purification of the zinc content as compared to a 14-fold increase in specific activity at a comparable level of purification.

Purity also was examined by various physical methods. Free-boundary electrophoresis demonstrated the protein to be essentially homogeneous with respect to charge. As shown in Fig. 2 a single boundary was observed with some skewing on the trailing edge. The electrophoretic mobility at pH 7.31 was calculated to be -0.68×10^{-5} $cm^2v^{-1}sec^{-1}$.

Only one band was observed following electrophoresis in cellulose acetate strips. The results for two preparations of the enzyme subjected to electrophoresis at pH 7.52 are presented in Fig. 3. The position of the protein

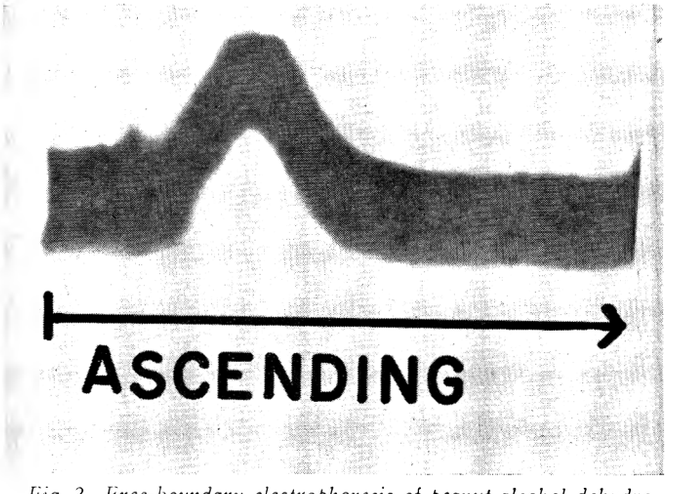

*Fig. 2. Free-boundary electrophoresis of peanut alcohol dehydrogenase. Schlieren photograph of the ascending boundary taken after 14,100 sec at a field strength of 7.4 volts cm^{-1}. The buffer was 01*M *phosphate (pH 7.31), which had been dialyzed against the protein solution.*

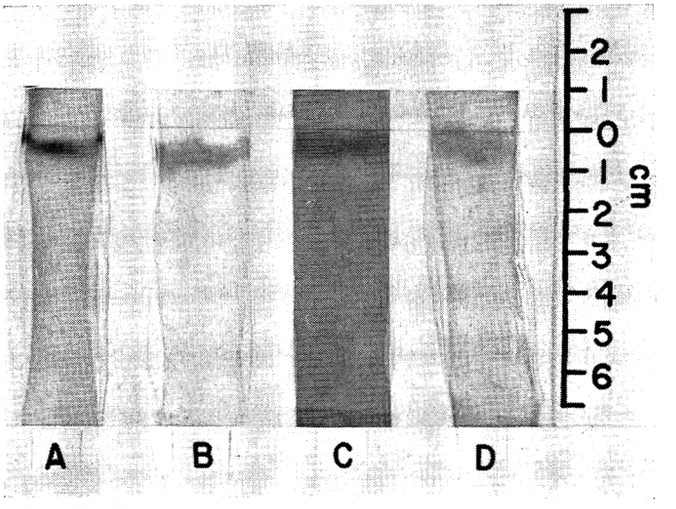

Fig. 3. Cellulose acetate zone electrophoretograms of peanut alcohol dehydrogenase. The protein was subjected to electrophoresis in phosphate buffer (pH 7.52, 0.05 Γ/2) for 60 min using a current of 1mA per strip and a potential of ca. *190 V. Strips A and C are protein stains from two independent isolations; B and D are the corresponding activity stains.*

and activity bands conclusively identifies the activity with the purified protein.

An illustration of the results obtained by sedimentation velocity ultracentrifugation using two different protein concentrations is given in Fig. 4. A single boundary was observed for both cases, demonstrating the size homogeneity of the preparation. The curvature at the top and bottom of the solution columns is due to a redistribution of the high concentrations of buffer salts.

DISCUSSION

A CONSIDERABLE DEGREE of purification of alcohol dehydrogenase from peanut kernels was achieved. Comparison of electrophoretic and ultracentrifugal data suggests that the degree of purity is comparable to that obtained for yeast and liver alcohol dehydrogenase (Sund *et al.*, 1963).

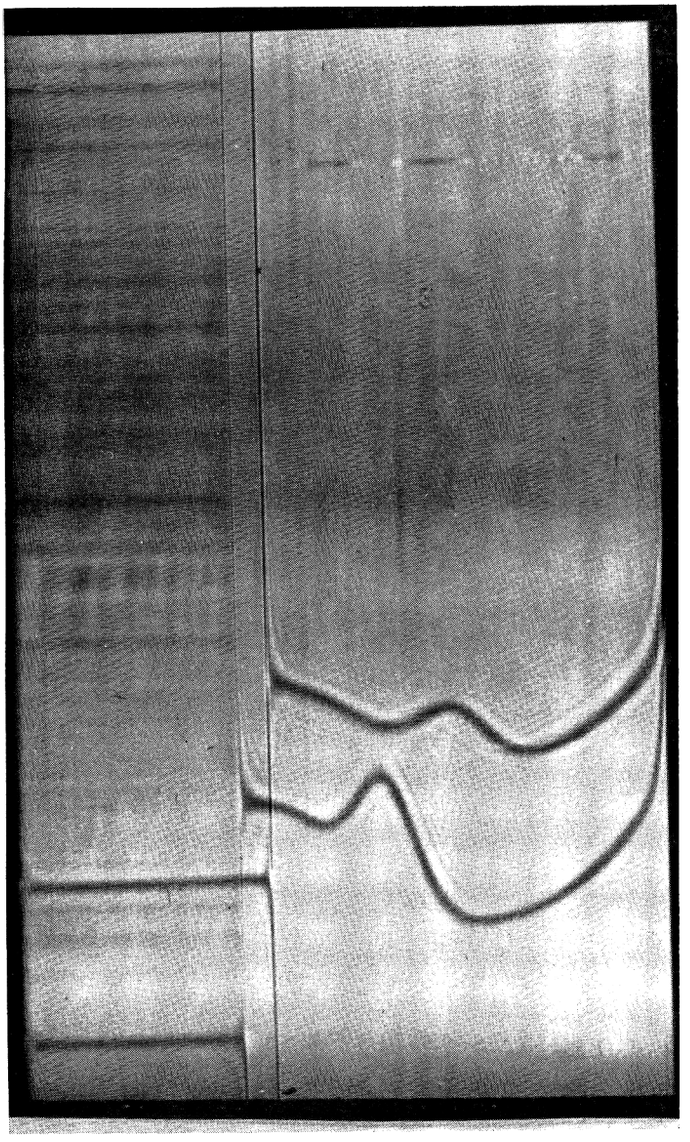

*Fig. 4. Velocity sedimentation of peanut alcohol dehydrogenase. Schlieren photograph of the sedimenting boundaries after 72 min at a rotor speed of 59,780 rpm and a temperature of 19°C. The upper curve represents the pattern for a protein concentration of 7.3 mg/ml and the lower curve that for a concentration of 14.6 mg/ml. The buffer was 0.1*M *phosphate, 0.4*M *NaCl, 0.01*M *cysteine, and 0.001*M *mercaptoethanol (pH 6.60).*

Alcohol dehydrogenase from higher plant sources has not been greatly purified. Stafford *et al.* (1953) reported some characteristics of a crude extract from wheat germ. Alcohol dehydrogenase has recently been isolated and characterized from pea seedlings by Suzuki (1966). Unfortunately no criteria for purity were presented. The specific activities reported indicate that the purification was less than two-fold. The method of isolation by Suzuki (1966) was similar to that reported in this communication. Our results suggest that a similar degree of purification was obtained at a comparable step in the isolation procedure.

The concomitant purification of zinc and enzyme activity indicates that the second Sephadex column yielded a zinc content of 1.5 g-atoms per 112,000 g of protein. Since increased specific activity was obtained for the 2-ml fraction containing the highest concentration of protein and activity, the true zinc content might be greater than this value.

Swaisgood *et al.* (1968) have investigated some of the physical and chemical properties of peanut alcohol dehydrogenase. The development of a method for obtaining alcohol dehydrogenase from a higher plant has thus provided a new source of this enzyme.

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GESTUR JOHNSON, BRENDAN J. DONNELLY and DUANE K. JOHNSON
Department of Chemistry, Colorado State University, Fort Collins, Colorado

The Chemical Nature and Precursors of Clarified Apple Juice Sediment

SUMMARY—Although considerable work has been done on the sediment sometimes formed in clarified apple juice upon storage, the sediment itself has not been completely characterized as to its chemical composition and the precursors involved. The fact that the sediment yielded phloroglucinol and protocatechuic acid upon alkali fusion and amino acids upon acid hydrolysis indicated that the sediment was a polymeric phenolic-protein complex. The variable nitrogen, mineral and ash contents of different sediments and the variable amino acid composition of the protein fraction in conjunction with the behavior of the sediments on Sephadex gel columns indicated the heterogeneous nature of this material. Polyamide thin-layer chromatography and colorimetric analyses have shown that leucoanthocyanidins and catechins are the main precursors of the polymeric phenolic component. Chlorogenic acid appears to play an insignificant role in sediment formation. The amino acid composition was determined by the use of an amino acid analyzer and the mineral content was estimated, quantitatively, by means of an atomic absorption spectrophotometer.

INTRODUCTION

CLOUDING FOLLOWED BY SEDIMENT FORMATION sometimes occurs during storage of clarified apple juice. The nature of this problem was discussed in detail by Neubert *et al.* (1944) whose investigations included work on the chemical nature of the sediment. They concluded that the sediment was probably a phlobaphene, a substance derived by heating condensed tannins with dilute acid. Qualitative chemical tests performed by them showed the absence of nitrogen, sulfur, halides and phosphates. Qualitative analysis of the ash showed the presence of iron and copper but no calcium.

As early as 1908, Kelhofer reported on the chemical nature of sediment formed in fermented pear juice. He concluded that the sediment contained protein, pectin and oxidized tannins.

In recent years, there has been a vast improvement in techniques and instrumentation that can be applied to a problem of this kind. The work we are reporting here will be limited to the chemical characterization of the sediment and the precursors involved in its formation. The problem of clouding and sediment formation is not strictly confined to clarified apple juice but exists in both the wine and beer industries.

Since the work of Neubert *et al.* (1944), no further work has been done to elucidate the chemical nature of the apple juice sediment. However, Letzig *et al.* (1963) investigated the nature of the sediment from "cloudy pressed apple juice" and concluded that the cloudy substances were a heterogeneous complex consisting of protein, polyphenolic matter, fragments of cell wall and nucleus and other ingredients. This would not be the same as the sediment formed in clarified apple juice.

EXPERIMENTAL

Source and isolation of sediment

Sediments were obtained from various sources; some from juices purchased on the retail market, others by storing various commercial juices at a temperature of 90 to 100°F for 3 to 11 months. The sediment was isolated by centrifugation of the juice using a Sharples centrifuge (Type T-41-24-8R) at approximately 40,000 rpm. The sediment was then removed and washed and again centrifuged at 10,000 rpm using a Serval angle centrifuge. This washing procedure was repeated twice.

Potassium hydroxide fusion

A 25 mg sample of sediment was added to 2 g of molten potassium hydroxide (ca. 300°C) and allowed to react at this temperature for 5 min. The cooled mass was dissolved in 5 ml of water and then acidified with conc. HCl. The resulting solution was extracted with 2 20-ml portions of diethyl ether. The combined ether extracts were concentrated to dryness under reduced pressure in a rotary evaporator. The residue was dissolved in 2 ml of 80% ethanol. The phenolic compounds were separated by polyamide thin-layer chromatography using 200 by 200 mm glass plates. Two solvent systems were used: (1) ethyl acetate-methanol-formic acid-water (70:30:10:10) and (2) ethanol-water-acetic acid (50:50:5). The phenolic compounds were detected by spraying the plates with diazotized sulfanilic acid.

C, H, N and ash analyses

The carbon, hydrogen, nitrogen (Kjeldahl) and ash contents of the sediments were determined by a commercial laboratory. (Galbraith Laboratories, Inc., Knoxville, Tenn.).

Separation of polyphenols in apple juice by thin-layer chromatography

One hundred ml of apple juice containing 5 g NaCl were extracted with 3 successive 35-ml portions of n-butanol. The butanol extracts were combined and evaporated to dryness under reduced pressure in a rotary evaporator. The residue was dissolved in 10 ml of 80% ethanol. The polyphenols were separated by two-dimensional polyamide (Woelm) thin-layer chromatography. After applying 20 μl of the ethanol solution, the chromatographs were developed by two solvent systems, (1) ethyl acetate-methanol-formic acid-water (70:30:10:10) and (2) ethanol-water-acetic acid (50:50:5). The polyphenols were detected by spraying the plates with diazotized sulfanilic acid, a generally suitable reagent for phenolics. The direct recognition of leucoanthocyanidins involved the use of toluene-sulfonic acid-reagent after the method of Roux (1957).

Mineral analyses

Twenty-five mg samples of the sediments were prepared for Cu, Ca, Mg, Fe and K analyses by digesting for 30 min with 5 ml of 70% $HClO_4$-conc. H_2SO_4-conc. HNO_3-2% $Na_2MoO_4 \cdot 2H_2O$ in the ratio 3.5:1:15:1. After digestion, the solutions were diluted 50 times with a 1.11% lanthanum solution (as lanthanum oxide) containing 1% H_2SO_4. The above solution was analyzed using a Perkin-Elmer 303 Atomic Absorption Spectrophotometer.

Amino acid analyses of sediments

Fifty mg samples of the dried sediments were heated under reflux with 10 ml of 6*N* HCl for 24 hr, cooled and filtered through Whatman No. 1 filter paper. The filtrates were concentrated to dryness under reduced pressure in a rotary evaporator. The residue was dissolved in 5 ml of water and concentrated to dryness as above. This process was repeated three times. The residue was taken up with the proper buffer and then the amino acids were determined using a Beckman model 120B or a Technicon amino acid analyzer.

Fractionation of sediment by gel filtration

Twenty-five mg of sediment was dissolved in 4 ml of 0.1*N* NaOH. The solution was adjusted to pH 7.0 with conc HCl and centrifuged to remove any slight amount of insoluble material. The sample was then passed through a Sephadex G-25 chromatographic column (2.5 × 45 cm) and eluted with deionized water at a flow rate of 60 ml per hr. The eluted fractions were collected, lyophilized and weighed. The excluded fraction from this column was passed through G-50 and the excluded fraction thus obtained was passed through G-75. Other samples of a sediment were eluted through G-100 and G-200 separately.

Analysis of juice

The total polyphenols were determined by the method as modified by Rosenblatt *et al.* (1941) and the leucoanthocyanidins by the method of Swain *et al.* (1959).

RESULTS AND DISCUSSION

Chemical nature of sediment

The results obtained at least partly substantiated the work of Neubert *et al* (1944). They concluded that the sediment was a rather inert substance, the properties of which resembled those of phlobaphene, a polymeric phenolic material. Our results showed that the sediment was at least partly phenolic in nature. The fact that the sediment particles in a water suspension turned blackish upon the addition of ferric chloride solution and turned more reddish brown upon heating with a mineral acid was an indication of the phenolic nature of the material. Furthermore, upon KOH fusion, two phenolic compounds were readily detected using polyamide thin-layer chromatography. The compounds were identified as protocatechuic acid and phloroglucinol. They had the same Rf values and gave the same color reactions with diazotized sulfanilic acid as the known compounds. The conspicuous absence or the low level of leucoanthocyanidins (proanthocyanins) and the catechins in commercially processed clarified juice suggested that these compounds were the precursors of the polymeric phenolic fraction of the sediment.

Table 1. Changes in total polyphenols and leucoanthocyanidins during processing of apple juice.

Sample description	Folin-Denis[1] mg[2]/100 ml juice	Leucoanthocyanidins O.D.[3] (515 mμ
Fresh juice[4]	120.7	0.2756
Regular juice from press	46.3	0.0223
Regular final product	41.5	0.0304

[1] After Rosenblatt *et al.* (1941).
[2] d-catechin (anhydrous) used as standard.
[3] Optical density—Evelyn colorimeter.
[4] Juice prepared with no enzymic oxidation from a sample of apples used in the processing.

Results of analyses of the changes in total polyphenols and in leucoanthocyanidins during different stages of processing showed that practically all of the losses in these compounds occurred during milling and pressing and were due to enzymic oxidation. The analyses of the juice from the press and of the final product are given in Table 1. Thin-layer chromatograms as shown in Figs. 1 and 2 clearly indicate that the largest losses occurred in the leucoanthocyanidins and the catechin fractions. In Fig. 1 the leucoanthocyanidins are in the area designated as number 1 and the catechins (d-catechin and l-epicatechin) as number 2. Spot 3 is chlorogenic acid and spots 4 and 5 were identified as phloretin glycosides by previously developed methods (Johnson *et al.*, 1963). Spot 4 is phlorizin and spot 5 is xylosylglucoside of phloretin.

From these results it would appear that the leucoanthocyanidins and the catechins are the principal precursors of the polymeric phenolic material formed during the milling and pressing stage. The polymeric material remaining after the clarification step not only contributes to the color of the apple juice but probably is the immediate precursor of the phenolic fraction of the sediment, which could form during storage under certain conditions.

Although chlorogenic acid is one of the principal poly-

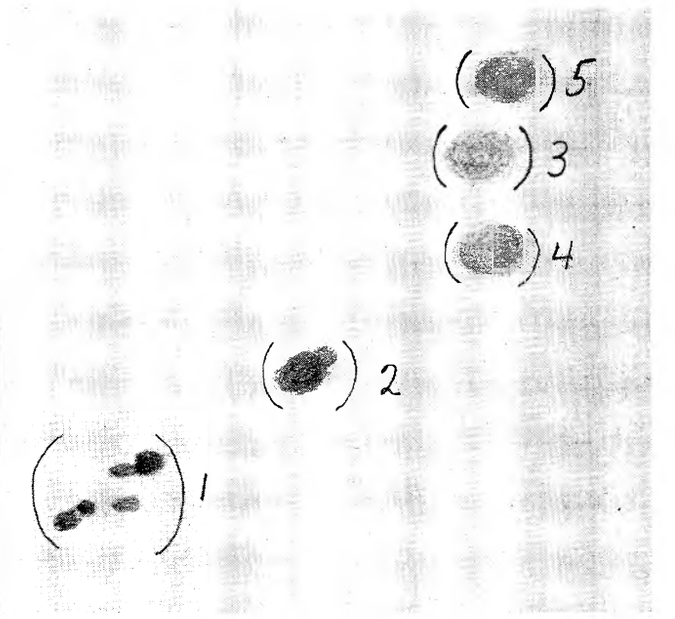

Fig. 1. Thin-layer chromatogram of n-butanol extract of fresh apple juice prepared without oxidation. (1) leucoanthocyanidins; (2) catechins; (3) chlorogenic acid; (4) phlorizin; (5) xylosylglucoside of phloretin.

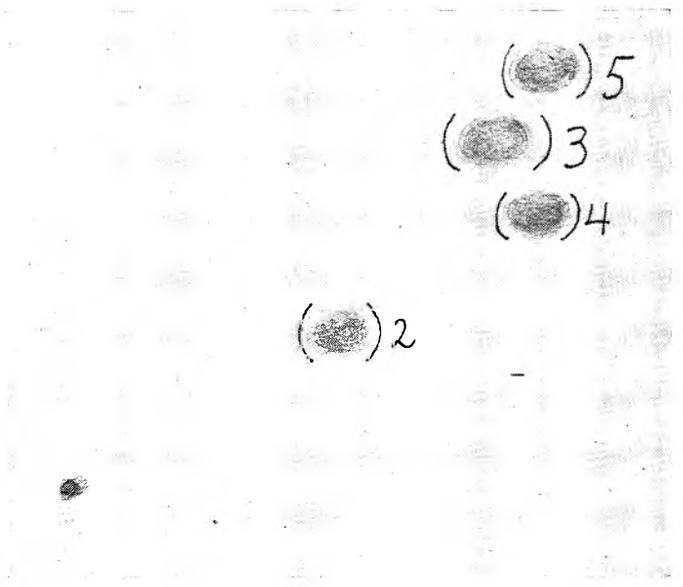

Fig. 2. Thin-layer chromatogram of n-butanol extract of apple juice from the press.

phenols occurring in apples, it is doubtful that it makes a contribution to sediment formation. This conclusion is based on the fact that chlorogenic acid is retained better in the final product juice than the leucoanthocyanidins and catechins and the fact that after hydrolysis of the sediment with 6*N* HCl only traces of quinic acid were detected by paper chromatography as described by Wright *et al.* (1960).

Neubert *et al.* (1944) reported a negative qualitative nitrogen test using Prussian Blue reaction following sodium fusion. This nitrogen test was found to give only a slightly positive test for some sediments and a definite test for others. However, paper chromatography of all HCl hydrolyzed sediments showed the presence of amino acids. This indicated that protein was associated with the polymeric phenolic fraction in some form of a polymeric phenolic-protein complex.

The heterogeneity of this complex became obvious upon analysis of various sediments for carbon, hydrogen, nitrogen and ash. The results, as shown in Table 2, reveal a large variation in both nitrogen and ash content.

Table 2. Analyses of apple juice sediments.

Sample description	Sediment mg/l	% C	% H	% N	% Ash
Brand A Sediment present when received	12	52.01	5.08	3.07	4.94
Brand A No sediment when received; held 4 months at 90°F	69	51.40	4.55	1.39	7.45
Brand B Sediment present when received; held additional 5 months at 70°F	14	47.27	4.82	3.22	9.97
Brand B No sediment when received; held 5 months at 90°F	17	46.52	5.00	2.99	9.16
Brand B No sediment when received; held 9 months at 100°F	10	54.02	5.14	2.43	4.18
Brand C No sediment when received; held 3½ months at 90°F	17	47.28	4.63	3.16	11.38
Brand C No sediment when received; held 9 months at 100°F	9	51.19	5.05	4.90	5.38
Brand D No sediment when received; held 9 months at 100°F	70	55.53	5.01	1.10	1.80

Amino acid analysis

Not only was the protein content variable, but the amino acid composition varied with the different sediments (Table 3). Sediments 1 and 2 are both from juices clarified with gelatin. Sediment 3 was clarified with nylon −66 powder (Polypenco). It was reasoned that since all the protein in this particular sediment had to come from apple protein, it was possible that in the other two sediments at least part of the protein could have come from residual gelatin. However, a comparison of the amino acid composition of the different sediments did not seem to support this hypothesis.

Fractionation of sediment by Sephadex gels

The results of fractionation of three sediments on three different Sephadex gels are illustrated in Table 4. The results obtained for two of the sediments were quite similar, but in the case of sediment from Brand A a greater portion was excluded by Sephadex G-75. The fraction, which was completely excluded from G-25, was redissolved in water and eluted through G-50 and the excluded fraction from this column was then eluted through G-75. The percentage of each fraction excluded by the various gels is given in Table 4. Of the fractions excluded by G-25, as low as 28 and as high as 70% were excluded by G-50. Thus this fraction had a molecular weight greater than 10,000. As low as 32% of this fraction (MW greater than 10,000) and as high as 78% had a molecular weight greater than 50,000. Tied with this fraction is also some material with a molecular weight range of 5,000 to 10,000 as was evidenced by the diffused material on the Sephadex G-50 column.

Sediment from Brand A was also eluted through G-100 and G-200 separately. Approximately 28% was excluded by G-100 indicating that this portion had a molecular weight greater than 100,000. Only a trace of material was excluded by G-200 showing that practically none of the sediment had a molecular weight greater than 200,000.

Table 3. Amino acid composition of sediment protein.

Amino Acid	Sediment 1 (Brand A)[1]		Sediment 2 (Brand C)[1]		Sediment 3 (Brand C)[2]	
	μmoles/ mg sed.	μmoles/ mg protein	μmoles/ mg sed.	μmoles/ mg protein	μmoles/ mg sed.	μmoles/ mg protein
Lysine	0.007	0.080	0.016	0.083	0.006	0.050
Histidine	0.003	0.034	0.002	0.010	0.012	0.101
Arginine	0.004	0.046	0.009	0.047	0.008	0.067
Aspartic Acid	0.023	0.264	0.034	0.176	0.039	0.330
Threonine	0.012	0.138	0.017	0.088	0.025	0.211
Serine	0.016	0.184	0.024	0.125	0.027	0.228
Glutamic Acid	0.032	0.945	0.028	0.145	0.037	0.313
Proline	0.012	0.138	0.053	0.275	0.027	0.228
Glycine	0.039	0.449	0.083	0.431	0.066	0.558
Alanine	0.016	0.184	0.037	0.192	0.034	0.287
Half Cystine	0.007	0.080	0.005	0.025	0.014	0.118
Valine	0.010	0.115	0.009	0.047	0.019	0.160
Methionine	0.001	0.011	trace	trace	0.001	0.085
Isoleucine	0.008	0.093	0.005	0.025	0.012	0.101
Leucine	0.011	0.126	0.008	0.041	0.017	0.144
Tyrosine	0.004	0.042	0.001	0.005	0.006	0.050
Phenylalanine	0.004	0.051	0.003	0.015	0.008	0.067

[1] Clarified with gelatin
[2] Clarified with nylon

Table 4. Fractionation of sediment by Sephadex gel filtration.

Sample	Gel	% Excluded
Brand A		
Sediment	G-25	62
Fraction excluded from G-25	G-50	70
Fraction excluded from G-50	G-75	78
Brand C		
Sediment	G-25	57
Fraction excluded from G-25	G-50	27
Fraction excluded from G-50	G-75	38
Brand D		
Sediment	G-25	68
Fraction excluded from G-25	G-50	28
Fraction excluded from G-50	G-75	32

Mineral composition

Analyses of several sediments for Ca, Cu, Fe, Mg and K by atomic absorption spectrophotometric methods showed a wide variation as indicated by the results shown in Table 5. Neubert *et al.* (1944) reported a negative qualitative test for calcium, but the above test indicated the presence of variable amounts of this element. The excluded fraction from Sephadex G-25 was found to be higher than the original sediment in all the elements determined although the ash content was lower. Here again the variability in the mineral composition is indicative of the heterogeneous character of the sediments.

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Table 5. Mineral content of sediments.

Sample	Ca ppm	Cu ppm	Fe ppm	K ppm	Mg ppm
Brand C	600	3,000	1,800	500	150
Brand B	40,000	2,000	1,300	1,300	300
Brand D	800	1,100	1,000	1,400	300
Brand D	700	700	800	600	160
Fraction excluded from G-25 (D)	2,000	1,500	1,200	1,200	320

A. H. ROUSE
University of Florida, Citrus Experiment Station
Lake Alfred, Florida 33850

R. A. DENNISON
Department of Food Science, University of Florida
Gainesville, Florida 32601

Gamma Radiation Effects on the Pectic Substances in Citrus Fruits

SUMMARY—Exposure of citrus fruits to gamma radiation at doses of 150 and 300 krad increased the water- and oxalate-soluble pectins and decreased the protopectin in component parts of Valencia oranges and Duncan grapefruit. The quantities of water-soluble pectin and protopectin in the peel and membrane were affected the most by irradiation. Degradation of the pectins as measured by jelly grade increased in all components of the irradiated fruits. The greatest decrease in jelly grade occurred in the March samples when the Brix/acid ratio was least. Methoxyl contents of the pectins from the peel of oranges and grapefruit decreased, while this characteristic decreased only slightly in pectins from the membrane of irradiated fruits. Pectinesterase activity in the peel of both oranges and grapefruit decreased with increased dosages, while the activity in the membrane of both fruits increased.

INTRODUCTION

EXPERIMENTALLY, ORANGES AND GRAPEFRUIT have been gamma and electronically irradiated primarily to try to reduce decay incidence as a means of extending their shelf life. However, undesirable side-effects of radiation results in softening of the peel tissue. Grierson *et al.* (1965) treated Valencia oranges and Marsh grapefruit with dosages ranging from 0 to 450 kilorad (krad) and reported severe peel injury and considerable damage to the fruit. Dennison *et al.* (1966) stated that citrus fruits examined showed irradiation-induced peel injury and the injury was greater as the radiation doses increased or as the storage temperature and duration of time increased.

The firmness of softening of citrus peel depends upon the chemical state of the pectic substances in the fruit tissues. During the maturation and/or ripening of many fruits, the pectic substances of the middle lamella undergo chemical changes which cause the softening of the tissues (Kertesz 1951). Rouse *et al.* (1962a) reported the changes of pectic substances in Valencia oranges during their maturation cycle in which protopectin decreased and water-soluble pectin and pectates increased with ripeness of the fruit.

Several investigators (McArdle *et al.*, 1956; Kertesz *et al.*, 1964; Somogyi *et al.*, 1964; Massey, Jr. *et al.*, 1964) observed not only the changes in the pectin fractions, but also the depolymerization of the pectins in irradiated fruits and vegetables. Their method for measuring pectin degradation was by the loss of viscosity. Kertesz *et al.* (1956) and Wahba *et al.* (1963) subjected aqueous pectin solutions to ionizing radiations at various dosages to determine the degradation of the pectin by the decrease in viscosities.

Valencia oranges and Duncan grapefruit exposed to 150 and 300 krad increased the water-soluble pectins in the extracted juices from 60 to 488% (Rouse *et al.* 1966). Juices from such irradiated oranges after storage for 3 weeks at 40°F usually had greater amounts of water-soluble pectin.

The present study was undertaken to determine the quantitative changes of the pectic fractions and the pectinesterase activity in the component parts of gamma irradiated Valencia oranges and Duncan grapefruit. Also, the effects of irradiation upon the characteristics of the pectins (such as jelly grade, methoxyl content, and jelly units) extracted from the fruit components were evaluated.

EXPERIMENTAL PROCEDURES

Preparation of samples

Valencia oranges and Duncan grapefruit were harvested from Experiment Station plots and hand-washed the day before irradiation. Orange samples were irradiated March 15, April 19, and May 24, 1966. Grapefruit samples were irradiated March 15 and May 24, 1966.

The fruits were irradiated in the Mark III cobalt-60 Food Irradiator located at the University of Florida, Gainesville. Dose rates for this unit were 3 krad/min for the side cell and 5.2 krad/min for the center cell. Samples were placed into the chamber and lowered into the pool for a period of time calculated to give the required dosage for the particular treatment. Controlled temperature in the chamber was 55°F, with a continuous flow of air at 20 liters/min during the irradiation period. The doses were 0 (control), 150, and 300 krad.

Twenty oranges and 10 grapefruit of treated and untreated fruits were used in preparing the samples of component parts. Peel, membrane, pressed juice sacs, and seeds were separated by hand and individually comminuted in an Osterizer.

Procedures in preparing the component parts for pectinesterase (PE) activity and for alcohol-insoluble solids (AIS) were as described by Rouse *et al.* (1962a).

Methods of analysis

Pectinesterase activity was measured essentially by the method of MacDonnell *et al.* (1945) as modified by Rouse *et al.* (1955). Units of activity are expressed by the symbol (PEu)/g, which represents the milliequivalents of ester hydrolyzed per min per g of dry tissues. These units are multiplied by 1,000 for easy interpretation.

Changes in the 3-pectic fractions were determined by progressive extractions with distilled water, 0.5% ammonium oxalate, and 0.05 *N* sodium hydroxide, and each of

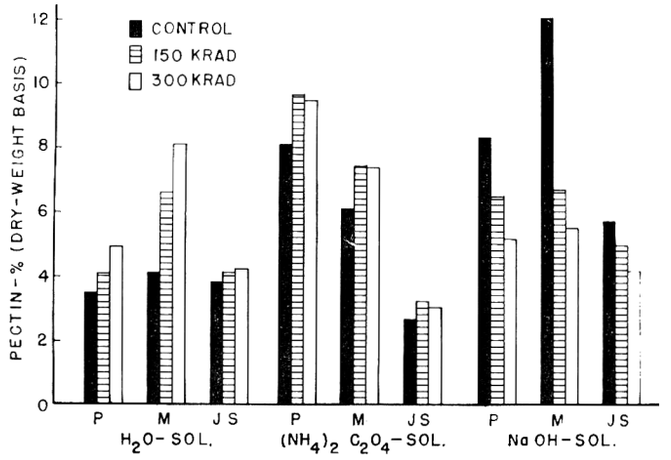

Fig. 1. Effects of gamma radiation on the pectic fractions in component parts of Valencia oranges. Each bar represents the average value of 3 samples. P = peel; M = membrane; JS = juice sacs.

these fractions was analyzed for anhydrobalacturonic acid (AGA) as previously described by Rouse *et al.* (1962a). The AGA is reported as percentage of pectin on a dry-weight basis (Figs. 1, 2).

Pectins used for determining jelly grades and other characteristics were extracted from the prepared AIS of peel, membrane, and juice sacs with distilled water and "Zeo-Karb" H at 90°C for 1 hr (Rouse *et al.* 1962b).

Yield of pectin is based upon the amount of alcohol precipitate obtained from the AIS of the component and the purity of the precipitate was determined as percentage of AGA (Rouse *et al.*, 1955).

Jelly grade of the pectin was determined by measuring the jelly strength of standard 65% soluble solids jellies with the Delaware Jelly-Strength Tester (Baker, 1938; Baker *et al.*, 1948).

Methoxyl contents of the pectins were determined by the procedure of Owens *et al.* (1952). Data for methoxyl contents were calculated on an AGA basis.

Jelly units is the resulting figure when yield of pectin is multiplied by jelly grade. It indicates the amount of sugar that can be gelled by one unit of raw material (Myers *et al.*, 1931.)

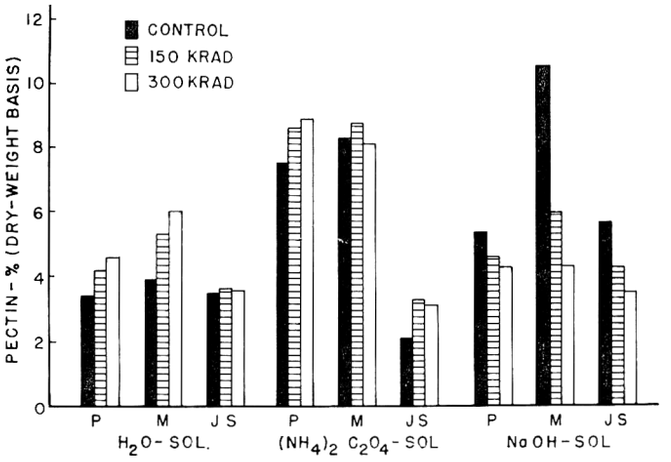

Fig. 2. Effects of gamma radiation on the pectic fractions in component parts of Duncan grapefruit. Each bar represents the average value of 2 samples. P = peel; M = membrane; JS = juice sacs.

RESULTS AND DISCUSSION

Pectic fractions

The first experiment revealed the effects of irradiation on the quantity of water-, ammonium oxalate-, and sodium hydroxide-soluble pectic fractions in peel, membrane, and juice sacs of both the controls and of the treated Valencia oranges and Duncan grapefruit (Figs. 1, 2). The bar graphs represent the average values of each pectic fraction from similar components of the 3 orange samples and of the 2 grapefruit samples. The water- and oxalate-soluble pectins in the orange components (Fig. 1) increased when the fruit was irradiated at 150 and 300 krad, but the sodium hydroxide fraction (protopectin) decreased with similar dosages of irradiation. Also, the water-soluble pectins increased as the dosage was increased, while the protopectin decreased with increased dosage. The 3-pectic fractions in the pressed juice sacs exhibited the least change.

The pectic fractions in the component parts of irradiated Duncan grapefruit (Fig. 2) show similar changes to Valencia oranges in the quantity of pectins when grapefruit were irradiated. Water-soluble pectin and protopectin from the membrane of both oranges and grapefruit were effected the most by irradiation. Acceleration of the pectic changes by radiation resulted in more rapid softening of the fruit than that which normally occurs during the maturation cycle.

Data on the 3-pectic fractions in the seeds of oranges and grapfruit are not presented, but they revealed that only the water-soluble fraction increased, with only a minor decrease in both the oxalate fraction and protopectin.

Characteristics of pectins

Sufficient quantities of pectins were extracted from the prepared AIS of the component parts of oranges and grapefruit to determine irradiation effects upon jelly grades and methoxyl contents. Jelly grade measurements were used to evaluate the depolymerization of the pectin molecule. Jelly grades of pectins were definitely lowered in the component parts of treated fruits when compared to the controls (unirradiated fruit) and in most instances, grades decreased with increased dosages (Tables 1, 2, 3). This further indicated that radiation, by accelerating the degrading of the pectin, caused fruit softening similar to that which takes place during the normal maturation cycle. The greatest decrease in the jelly grades of the pectins was found in the peel and membrane of both oranges and grapefruit when the Brix/acid ratio of the juices was least. This occurred in the samples picked in March.

These results (Tables 1, 2, 3) seem to be somewhat similar to those of Kertesz *et al.* (1956), who showed that sugars acted as protectants against radiation when added to pectin solutions which were irradiated. Their data would indicate that the more mature the fruit the less effect of irradiation on pectin grade. Ting *et al.* (1961) reported that total sugars in orange and grapefruit peel increased with maturity of the fruit.

Methoxyl contents of pectins extracted from the peel of oranges and grapefruit irradiated at 150 and 300 krad usually decreased with dosage (Table 1), while pectins extracted from the membranes of these fruits indicated only slight decrease in the methyl ester (Table 2) as compared to the pectin from unirradiated fruit. Pectins from juice sacs showed no definite pattern in their methoxyl contents (Table 3). Kertesz *et al.* (1956) irradiated dry pectins and pectin solutions, but found no significant change in the methyl ester contents. However, their highest dosage was 233.7 krad.

The methoxyl contents of the pectins were calculated on the basis of AGA which ranged from 74.7 to 89.4% in the components of oranges and 79.0 to 96.0% in the components of grapefruit. Radiation dosages did not effect the purity of the alcohol precipitates as AGA. Pectins from the membrane always contained the highest AGA and from the juice sacs the lowest AGA.

Jelly units varied with the grade of the pectin and were higher in both the peel and membrane than that in the juice sacs of unirradiated fruit. Juice sacs of oranges sampled 3/15 and 5/24, which received 150 krad, contained slightly greater jelly units than those from the control fruit because of the increased yield of pectin.

Pectinesterase activity in the peel of irradiated oranges and grapefruit decreased, while it increased in the membrane with dosage (Table 4). At the 150-krad dose, the PE activity dcreased in the juice sacs of both oranges and grapefruit and then increased at the 300 dosage. This same behavior again was exhibited in the seeds from grapefruit, but seeds from oranges increased in PE activity with dosage.

Table 1. Effects of gamma radiation on the characteristics of pectins extracted from the peel of citrus fruits.

		Valencia oranges				Duncan grapefruit			
Date of treatment	Doses of irradiation	Yield (%)	Jelly grade	Jelly units	Methoxyl content (%)	Yield (%)	Jelly grade	Jelly units	Methoxyl content (%)
3/15/66	0 (control)	22.4	337.6	75.6	11.13	23.5	302.3	71.0	11.05
	150 krad	24.6	256.4	63.1	10.15	23.4	252.9	59.2	9.99
	300 krad	27.5	222.6	61.2	9.91	25.4	237.2	60.0	9.84
4/19/66	0 (control)	23.0	252.4	58.1	10.73	.....	.....	.....	.....
	150 krad	24.1	231.3	55.7	10.29	.....	.....	.....	.....
	300 krad	22.8	218.8	49.9	10.33	.....	.....	.....	.....
5/24/66	0 (control)	23.5	262.1	61.6	11.06	23.5	285.7	67.1	11.36
	150 krad	24.2	248.1	60.0	10.47	24.4	276.6	67.5	10.30
	300 krad	25.7	234.6	60.3	9.99	25.1	258.4	64.9	9.90

Table 2. Effects of gamma radiation on the characteristicsof pectins extracted from the membrane of citrus fruits.

		Valencia oranges				Duncan grapefruit			
Date of treatment	Doses of irradiation	Yield (%)	Jelly grade	Jelly units	Methoxyl control (%)	Yield (%)	Jelly grade	Jelly units	Methoxyl control (%)
3/15/66	0 (control)	32.4	369.3	119.7	12.23	36.4	391.5	142.5	12.00
	150 krad	30.9	299.5	92.5	11.50	36.1	329.1	118.8	11.46
	300 krad	32.9	286.3	94.2	11.97	37.0	281.4	104.1	11.37
4/19/66	0 (control)	29.4	366.1	107.6	11.97	.....	.....	.....	.....
	150 krad	31.9	333.3	106.3	11.58	.....	.....	.....	.....
	300 krad	33.8	311.0	105.1	11.69	.....	.....	.....	.....
5/24/66	0 (control)	34.0	321.0	109.1	11.70	32.5	345.7	112.3	11.56
	150 krad	35.5	276.6	98.2	11.49	33.6	329.1	110.6	11.06
	300 krad	36.9	257.9	95.2	11.14	33.9	312.5	105.9	11.22

Table 3. Effects of gamma radiation on the characteristics of pectins extracted from the juice sacs of citrus fruits.

		Valencia oranges				Duncan grapefruit			
Date of treatment	Doses of irradiation	Yield (%)	Jelly grade	Jelly units	Methoxyl content (%)	Yield (%)	Jelly grade	Jelly units	Methoxyl content (%)
3/15/66	0 (control)	19.2	227.3	43.6	11.18	19.6	185.2	36.3	11.07
	150 krad	20.3	219.6	44.6	11.52	21.8	150.3	32.8	10.95
	300 krad	19.2	194.0	37.2	11.60	21.1	154.4	32.6	11.08
4/19/66	0 (control)	19.8	285.1	56.4	11.58	.....	.....	.....	.....
	150 krad	19.5	250.0	48.8	11.41	.....	.....	.....	.....
	300 krad	20.0	222.2	44.0	11.14	.....	.....	.....	.....
5/24/66	0 (control)	15.6	233.8	36.5	12.54	20.8	282.6	58.8	10.93
	150 krad	17.2	218.1	37.5	11.70	21.4	254.9	54.5	10.54
	300 krad	15.9	225.3	35.8	11.51	22.1	255.9	56.6	11.08

Table 4. Effects of gamma radiation in the pectinesterase activity[1] in the component parts of citrus fruits.

Component parts	Valencia oranges[2]			Duncan grapefruit[3]		
	Control	150 krad	300 krad	Control	150 krad	300 krad
Peel	52.5	35.2	37.2	51.1	43.3	41.5
Membrane	82.1	100.8	142.6	47.3	50.5	62.8
Juice sacs	294.7	278.7	337.3	213.9	181.6	199.9
Seeds	2.5	3.1	3.6	3.9	3.1	4.3

[1] Units per g of dry tissue $\times 10^3$.
[2] Average of 3 samples.
[3] Average of 2 samples.

Somogyi *et al.* (1964) found that PE in cherries increased in activity immediately after dosages of 200 and 500 krad, and 4 days after irradiation had a reduced activity. Their results could indicate several possibilities. First, the skin of cherries, receiving the full exposure of radiation, could have actually decreased in PE activity, while that in the fleshy part of the cherries increased. Secondly, the net result of radiation on PE activity of the whole pitted cherries, in which the flesh was the major part of the fruit, could have had greater activity actually resulting in an overall increase in PE activity. Similarly, if a composite were made of the component parts of oranges, the net result would be an increase in activity as the dosage was increased up to 300 krad.

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CHRISTIAN J. B. SMIT and EDWIN F. BRYANT
Research and Development Division, Sunkist Growers Inc., Corona, California

Ester Content and Jelly pH Influences on the Grade of Pectins

SUMMARY—A series of samples with percent esterification ranging from 73.7 to 28.6 was prepared under acid conditions at room temperature from lemon peel pectin that had been precipitated with aluminum. Molecular weight, as determined by viscosity in 1% Calgon, and galacturonic acid content remained unaltered during demethylation while equivalent weight decreased. Setting time increased until an ester content of around 50% was reached. At lower ester values setting time decreased again.

Maximum jelly grades were obtained at an ester content of *ca.* 45%. However, at this ester level the pH at which a high jelly grade was obtained was very low and the grade decreased very rapidly as the jelly pH was increased to values above pH 2.2. The maximum grade obtained with a particular sample decreased and the pH at which this maximum grade could still be obtained increased as the ester content was increased above *ca.* 45%. Below this ester level, the maximum grade as well as the maximum pH at which this grade could be obtained decreased with ester content.

INTRODUCTION

COMMERCIALLY-PRODUCED PECTINS are used primarily to make jellies and jams. For this reason, they are standardized with respect to their ability to form a satisfactory jelly. Factors that influence gel-forming ability are consequently of great importance to both the producer and user of pectin. Apart from the factors involving gel making and gel testing procedures, characteristics of the pectin itself such as source, molecular weight and degree of esterification have an important influence on the firmness and texture of a jam or jelly.

The relationship between the degree of esterification of pectin and gel quality has been investigated by several workers in the past, such as Baker *et al.* (1941) and in more recent years by Doesburg (1950) and Pilnik (1964), who published detailed data on apple pectins. Such studies were usually conducted with specially selected samples, which differed in, among other things, ester content. Alternatively, samples were prepared by methylation or demethylation of pectin without direct evidence that molecular weights did not change in the process.

The present study reports data on a series of citrus pectins prepared from the same starting material. These samples differed only with respect to the ester content and were similar with regard to molecular weight and the presence of ballast materials.

EXPERIMENTAL

Preparation of samples

A sample of 15 kg pressed aluminum precipitated pectin, prepared from lemon peel, was shredded and left overnight in 75% isopropanol to harden. The alcohol was drained off and the pectin was passed through a grinder with a screen having openings of about 0.1 in. A first sample of 750 g was taken at this stage. This material was washed with 5% HCl in 60% isopropanol and residual acid was removed with 60% isopropanol. It was then washed with high proof isopropanol and dried in air.

The remainder of the ground pectin (7.7 kg) was suspended in 15 l 60% isopropanol containing 10% HCl and stirred for 30 min to remove aluminum. It was then filtered off and washed with a further 3 l 60% isopropanol. The washed material was added to a mixture of 9.6 l isopropanol, 5 l H_2O and 1.4 l conc. HCl, and stirring with a mechanical stirrer was started. Nine samples of *ca.* 700 g drained pectin were taken periodically over 10 days. These were washed with 60% isopropanol until free of chlorides, then finally rinsed with high proof isopropanol and air dried.

After drying the ash content was determined to check degree of aluminum removal during acid treatment. The ash content was in all cases below 0.1%, indicating satisfactory washing. The samples were then buffered by determining the percentage free carboxyl groups present and using half the amount of Na_2CO_3 necessary to give full neutralization of the pectin. The Na_2CO_3 was dissolved in 320 ml H_2O; 480 ml isopropanol was then added and the pectin suspended in this solution. This was left for an hour with occasional stirring before filtration, washing with isopropanol and drying in air.

Analytical procedures

Moisture, ash, percent methoxyl, degree of esterification, percent galacturonic acid, equivalent weight, molecular weight, jelly grade and setting time were determined as described by Smit *et al.* (1967). Viscosity was determined using an Ostwald-Cannon-Fenske viscosimeter. In order to determine the influence of jelly pH on jelly grade, five-glass batches of jelly were usually made. The jelly was poured into glasses containing varying amounts of 5*N* tartaric acid solution. Throughout, soluble solids were corrected to 65° Brix and all jellies having a sag outside of the 22–26% range were discarded. To make comparisons easier, all grade values were expressed on an ash- and moisture-free basis.

RESULTS AND DISCUSSION

DETAILED ANALYSES of the samples are given in Table 1. As the percent esterification decreased the setting time increased until an ester value of about 50% was reached. Hereafter, setting time decreased again very markedly. This agrees closely with the suggestion of Doesburg *et al.* (1960) that maximum setting times occur at about 50% esterification. Galacturonic acid content remained relatively constant during deesterification. There was a marked increase in viscosity at low ester levels (samples 8–10). At these low ester levels the pH and the amount of buffer used have a very big influence on viscosity (Kertesz, 1951) and the data obtained are thus not of much significance.

Molecular weights, determined by viscosity, remained

Table 1. Analyses of prepared pectins.

No.	Acid treatment (hr)	pH (1% sol)	cP Viscosity	% Moisture	% Ash	% Ester	% OCH_3[1]	% Gal. acid[1]	Eqv. wt[1]	Mol. wt. ÷ 1000	Set time (sec)
1	0	3.66	40.7	9.8	2.8	73.7	10.4	87.8	838	152	39
2	14	3.64	38.1	9.0	2.9	67.3	9.3	86.3	687	150	167
3	22	3.81	38.4	10.1	3.2	65.9	8.9	84.9	661	153	211
4	39	3.87	38.1	9.9	3.8	61.9	8.5	85.5	595	154	290
5	64	3.90	38.1	10.7	4.3	56.1	7.6	84.0	527	158	400
6	88	3.87	36.5	11.4	4.5	51.2	7.2	87.9	452	156	460
7	135	3.91	47.2	11.7	5.1	43.7	6.1	87.0	396	157	440
8	183	3.98	124.1	11.6	5.9	36.1	5.1	88.3	344	161	390
9	201	4.09	90.1	13.2	6.3	32.6	4.7	88.8	324	152	320
10	229	4.10	194.8	12.7	6.9	28.6	4.1	89.5	304	153	150

[1] Values expressed on an ash- and moisture-free basis.

relatively constant during deesterification and it was concluded that no significant depolymerization had occurred under the conditions used.

The ash- and moisture-free grades obtained at different pH values for some of the pectins are given in Fig. 1.

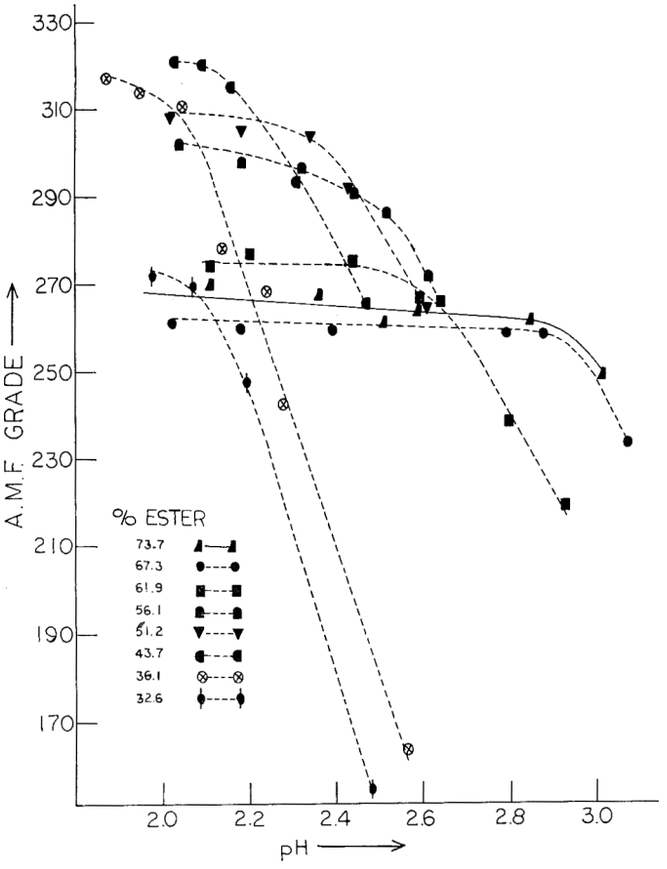

Fig. 1. Relationship between jelly grade and pH.

The starting material (sample 1) with an ester content of 73.7% had a slightly higher grade than that of the first two retarded samples (2 and 3) at pH values below 2.5. The first three samples with an ester content of 73.7%, 67.3% and 65.9% respectively showed little change in grade at pH values below 3 and only started losing grade rapidly above pH 3.0. As the ester content decreased further from one sample to the next the grade at low pH values increased while the pH at which maximum grades were obtained decreased.

The highest grade was obtained at pH 2.2 with a pectin having an ester content of 43.7%. However, this sample lost grade very rapidly as the jelly pH was increased to higher levels. With a further decrease in ester content the maximum grade obtained started decreasing again while the pH at which this maximum grade could be obtained decreased still further. A jelly could be made with sample 10 (28.6% ester) at pH 2.14, but it was not possible to obtain satisfactory jellies when the pH was increased above this point.

By extrapolation of the curves shown in Fig. 1, the relationship between jelly grade and pH at different ester levels could be calculated. These data are given in Table 2. This information agrees well with the findings of Doesburg (1950), who showed that jellies prepared at pH 2.1–2.3 from samples demethylated to values around 50% ester content gave higher grades than those prepared from samples with a higher ester content. There is also good agreement with Pilnik (1964), who showed that optimum grades are obtained at pH values around 2.2–2.4 and ester values below 55%. It is probably safe to assume that the general relationship between ester content, grade and jelly pH that have been developed in this report will hold true if other factors influencing grade, such as molecular weight, the presence of cations, or enzyme action, do not enter as variables.

Table 2. The relationship between jelly grade[1] and pH at various methoxyl levels.

pH	% OCH_3[1] 10.0	9.5	9.0	8.5	8.0	7.5	7.0	6.5	6.0	5.5	5.0
3.0	259	258	258	248	227	209	189	168	.....	.....	.....
2.8	261	258	259	259	257	252	241	227	210	.....	.....
2.6	262	259	259	270	282	288	283	272	254	220	175
2.4	263	259	260	273	288	298	305	305	293	265	236
2.2	263	260	261	274	291	302	311	317	319	313	297

[1] Values expressed on an ash- and moisture-free basis.

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DELPHA VENSTROM and JOHN E. AMOORE

Western Utilization Research and Development Division, Agricultural Research Service
U. S. Department of Agriculture, Albany, California 94710

Olfactory Threshold in Relation to Age, Sex or Smoking

SUMMARY—The individual olfactory thresholds of 97 persons toward 18 odorants were analyzed statistically. There is a significant logarithmic deterioration with age, the average loss of sensitivity being 50% in 22 years. Any influence of sex or smoking is negligible.

INTRODUCTION

AMOORE ET AL. (1967) recently completed an extensive series of measurements of individual human olfactory thresholds. The original objective of the experiments was the identification of a "primary odor" by the study of specific anosmia (Amoore, 1967). However, the data were further analyzed for any systematic differences in olfactory sensitivity among various subgroups of the population. Considering that the measurements included 18 odorants and were applied to 97 normal subjects, this survey is believed to be more comprehensive than any earlier study of these questions.

METHODS

A DETAILED DESCRIPTION of the experimental procedure is found in Amoore *et al.* (1968). The odorants (all rigorously purified) were the ten normal fatty acids from formic to capric; isobutyric acid; isovaleric, α-methylbutyric and trimethylacetic acids; isocaproic acid; isobutyl alcohol, isobutyraldehyde and isobutyl isobutyrate. The odorants were presented in aqueous dilution at controlled pH, in stoppered flasks. At each dilution the subject had to distinguish the two odorous flasks from three odorless controls (2/5 test; chance level of success 1 in 10). The dilution scale consisted of binary steps (successively halving the concentration of odorant).

The subjects were personnel of this laboratory. Two preliminary screening tests were applied (Amoore *et al.*, 1968), to eliminate any general anosmics, partial anosmics, and persons specifically anosmic to isobutyric acid and its congeners. These tests were not severe, resulting in the rejection of only about 4% of the people tested. The final working panel consisted of the first 97 persons who passed the tests; they were considered to have a normal sense of smell for the present purpose.

The units of measurement and calculation were binary dilution steps throughout ($\log_2$ scale). For each subdivision of the panel, and each odorant, we calculated the group mean olfactory threshold, standard deviation (SD), standard error of the mean (SEM), and the probability P of any observed difference between subdivisions by Student's t test. Values for $P > 0.05$ were regarded as insignificant. The "relative olfactory sensitivity" for each subject was also calculated; that is, his individual sensitivity towards a given odorant compared with the mean threshold for the whole panel, which was set at zero. His relative sensitivity was then averaged algebraically over all 18 odorants.

RESULTS

Age

The panel contained 35 persons under 40 years of age and 62 over 40. The younger group invariably achieved a lower threshold than the older group, and for 15 of the 18 odorants the differences were significant at the level $P = 0.05$ or less. The mean difference in threshold, averaged over all 18 odorants, was 1.15 steps in favor of the younger subjects.

The effect of age on the average relative sensitivity is shown as a scatter diagram in Fig. 1. Despite the marked intersubject variation, the general logarithmic decline in sensitivity with age is apparent, over the tested range of 20 to 70 years. The regression line has a slope of -0.046 $\log_2$ step per year (SE of estimate 1.01 step), which corresponds with a 50% loss of sensitivity in 22 years. The correlation coefficient is -0.47, and with 97 subjects the trend is highly significant ($P < 0.001$). However, it should be realized that little more than 20% of the total variance in individual olfactory sensitivity can be accounted for as a function of age.

Sex

The distribution of men and women was 48:49. For only one of the odorants did a significant difference in olfactory sensitivity appear between the sexes. The mean difference in threshold, algebraically averaged over all 18 odorants

Table 1. Loss of sensory acuity with age. Values in italics are recalculated by the present authors from data or graphs in original references.

Sense	Units of measurement	Rate of deterioration		50% loss (years)	Reference
		$\log_{10}$ per year	$\log_e$ per year		
Sight (night vision)	intensity (μLambert)	0.023	*0.076*	13	McFarland *et al.* (1955)
Sound (125–500 cps)	power (μWatt)	*0.020*	*0.066*	*15*	Hinchcliffe (1959)
Smell (18 odorants)	concentration (ppm)	0.0139	0.046	*22*	(This work)
Taste (sugar & salt)	concentration (%)	0.0105	*0.035*	*29*	Hinchcliffe (1958)
Touch (corneal)	weight (mg)	*0.0050*	*0.017*	*60*	Boberg-Ans (1956)

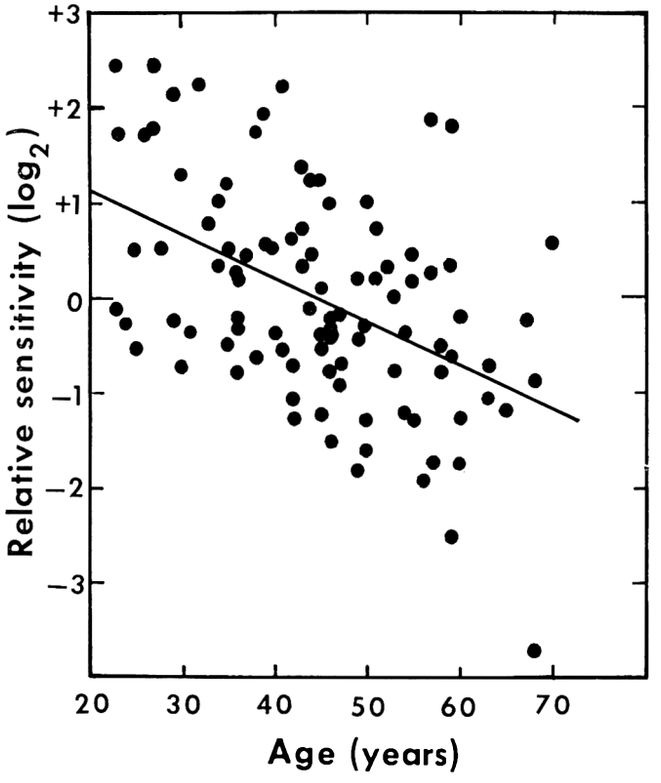

Fig. 1. Effect of age on olfactory sensitivity. The results were obtained for 97 observers tested with 18 odorous compounds. The slope of the regression line is −0.046 $\log_e$ step per year.

and corrected for age differential, was just 0.23 step in favor of the women.

Smoking

There were 18 tobacco users in the group. Significant differences were noted for only two odorants. The mean threshold difference (corrected for age) was merely 0.19 step in favor of the nonsmokers.

DISCUSSION

EVIDENTLY FOR THESE ODORANTS the influence of sex or smoking is negligible, and can be ignored for most practical purposes. However, the deterioration of the sense of smell with advancing years appears to be real. The observed decrease in olfactory acuity with age confirms the conclusions of three earlier studies, which employed serial dilutions of odorants in mineral oil. Fordyce (1961) and Joyner (1963) tested phenol, and Kimbrell *et al.* (1963) used *n*-butanol and isoamyl acetate. Presumably on account of inconsistencies in their results, none of these authors attempted to calculate an actual rate for the decline in sensitivity with age.

Fordyce (1961) found no effect of cigarette smoking but a possible decrease in acuity among pipe-smokers, whereas Joyner (1963) noted a significant decrease among smokers. Concerning the sex of the subject, Schneider *et al.* (1955), using air dilution of citral in a walk-in olfactorium, found a significantly higher olfactory sensitivity among women. The present work suggests that any slight advantage exhibited by non-smokers and by women is so small that it could become significant only in a very large series of tests.

Hinchcliffe (1958) demonstrated that the acuity of the sense of taste likewise declines logarithmically with age. The rate of deterioration, which was the same for both sucrose and sodium chloride, worked out at 0.0105 $\log_{10}$ step per year. On recalculation, this corresponds with a 50% loss of taste sensitivity in 29 years, which may be compared with the 22 years for 50% loss of smell sensitivity. This general decline in the senses of taste and smell with age could be relevant to domestic and institutional preferences and complaints regarding food flavors.

Hinchcliffe (1962) pointed out that deterioration with age applies qualitatively to all the special senses, apart from smell for which no data were then available. It is now possible to complete Hinchcliffe's compilation, and assemble the data quantitatively for comparison (Table 1). The logarithmic rates of deterioration are expressed in those physical units with which the threshold stimuli are conventionally measured. It is apparent that the sense of smell occupies an intermediate position within a wide range of sensory durabilities. The actual numerical values may be of interest for psychophysics and gerontology.

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TIBOR FULEKI [a] and F. J. FRANCIS
Department of Food Science and Technology
University of Massachusetts, Amherst, Massachusetts 01003

Quantitative Methods for Anthocyanins. 3. Purification of Cranberry Anthocyanins

SUMMARY—The evaluation of the various purification methods was based, in the order of their importance, on the recovery of individual and total anthocyanins and on the concentrating power. Amberlite CG-50 ion exchange resin was the best, but basic lead acetate was also satisfactory. Polyamide did not concentrate the anthocyanins and the use of neutral lead acetate resulted in poor recoveries.

INTRODUCTION

SEPARATION OF THE INDIVIDUAL ANTHOCYANINS (I Acys) is necessary before the quantity of each anthocyanin (Acy) can be determined. With fresh or frozen berries, the crude pigment extract may be separated without any purification. However, with products containing appreciable amounts of added sugar such as cranberry juice cocktail (CrJ) and sauce, the separation is not feasible without preliminary purification to remove or reduce the sugar content and concentrate the anthocyanins (Acys).

Various methods are described in the literature, such as solvent extraction; lead, mercury, and zinc acetate precipitations; polyamide and ion exchange resin and cellulose columns, for the preliminary purification of Acys present in solutions containing high concentrations of sugar. Since none of these methods was previously used for quantitative analysis of individual pigments, it was necessary to evaluate them from this viewpoint. This paper evaluates quantitatively the three most frequently used Acy purification methods, namely lead acetate precipitation, polyamide and ion exchange resin columns for the purification of Cr Acys.

In the method developed for the quantitative determination of I Acys (Fuleki *et al.*, 1968b) the total anthocyanin (T Acy) content was measured directly on the CrJ. The ratio of I Acys was determined after they were separated chromatographically. The purification treatment was carried out prior to the chromatographic separation. For this reason, the loss of Acys during purification would not result in an error as long as the ratio of individual pigments remains unchanged. Consequently, this paper is concerned with the various purification methods primarily on the basis of I Acy recovery.

LITERATURE REVIEW

Precipitation with lead acetate

The precipitation of Acys as lead salts is the oldest method used for the separation of Acys from the other plant constituents (Deibner *et al.*, 1963) and is still frequently used for the preliminary purification and concentration of Acys. Various salts or pseudosalts of bivalent lead have been used for the precipitation of Acys. An aqueous or alcoholic solution of neutral (normal) lead acetate, $Pb(C_2H_3O_2)_2$ is used most frequently. Solutions of the more basic lead subacetate (basic lead acetate) $Pb(C_2H_3O_2)_2 \cdot 2Pb(OH)_2$ are also commonly used. Other lead compounds such as lead nitrate (Braconnot, 1807) and lead hydroxide (Roach, 1958) have also been employed.

Anthocyanins are not the only group of compounds present in plant extracts which are able to form slightly soluble lead salts. Compounds possessing a free carboxyl (organic, phenolic, fatty and amino acids and proteins) or other nucleophilic groups, such as a phenolic hydroxyl group (flavonoids, tannins, etc.), are also precipitated by lead acetate. Since lead acetate will precipitate numerous other plant constituents as well as Acy, this method is not suitable for the absolute purification of Acy. Nevertheless, it is well suited for the preliminary purification of Acys present in aqueous or alcoholic plant extracts, because the most troublesome and plentiful impurities, the sugars, are not precipitated by this reagent.

In addition to the preliminary purification, this method transfers the Acy from an aqueous to an alcoholic solvent. The Acy is concentrated by using a minimum amount of alcohol and either hydrochloric or sulfuric acid to liberate the pigments from their lead salts. The precipitated lead salt formed upon the addition of the acid is removed from the Acy solution by centrifuging. Acetic acid is not used for the liberation of Acys from their lead salts because the resulting lead acetate is slightly soluble in alcohol and very soluble in water. The presence of lead acetate in the Acy solution would interfere with the further analysis of the sample.

The reaction between lead and Acys is not well understood. In contrast with the more stable, highly soluble, aluminum and ferrous iron chelates where covalent bonds are involved between the metal and the o-dihydroxyl group, the chemical bond between the heavy metal cation and the nucleophilic hydroxyl group is essentially of ionic nature. Due to the amphoteric character of Acy, the reaction between the Pb^{++} and Acy is pH-dependent.

Several authors (Stasunas, 1955; Geissman, 1955; Hayashi, 1962) attributed greater precipitating power to basic than to neutral lead acetate. Since the optimum pH for the precipitate formation is different for the various I Acys, some fractionation can be accomplished by adjustment of the pH (Thompson, 1959).

[a] Present address: Horticultural Products Laboratory, Horticultural Research Institute of Ontario, Vineland Station, Ontario, Canada.

According to Geissman (1955) and Hayashi (1962), the presence of adjacent free hydroxyl groups in the Acy molecule is favorable for the formation of insoluble lead salts. Consequently, glycosides of cyanidin, delphinidin and petunidin are precipitated at a lower pH than that of pelargonidin, peonidin or malvidin. This indicates that the phenolic hydroxyl group of Acys containing a free o-hydroxyl group is more acidic than those lacking such an end-group. This difference in reactivity between Acys is utilized for identification purposes by using lead acetate as chromogenic reagent (Fuleki *et al.*, 1967).

In grape juice and wine analysis, methods employing fractional precipitation of Acys using either basic lead acetate following the neutral lead acetate precipitation or neutral lead acetate with pH adjustments are widely used.

Considering the instability of Acys in alkaline media, it is unwise to increase the pH to a high level or to use basic lead acetate. According to Sengewald (1961) lead acetate might cause some degradation of Acy, particularly in a media which is not highly acidic. Onslow (1925) noted that lead acetate precipitation presents the same hazard as the use of alkali. According to Willstatter *et al.* (1913) the degradation of Acy in this case is due to excess of the alkaline reagent or to long exposure. Stasunas (1955), however, found that the average error in recovery of synthetic anthocyanidins added to red wines was only 4% (range 1.45–15.4) after basic lead acetate treatment.

Sengewald (1961) reported 92.5% recovery of hybrid red wine Acys after using neutral lead acetate and ammonia to precipitate the pigments. Somers (1966) found that malvidin-3-glucoside and the acylated pigments were only partially precipitated from grapes by neutral lead acetate and peonidin-3-glucoside not at all. Some loss of the more labile acylated pigments occurred when the precipitation was carried out with basic lead acetate (Somers, 1966) or in a slightly basic media (Ribéreau-Gayon, 1959). Storage of the pigments in their lead complex form resulted in complete loss of acylated anthocyanins (Somers, 1966).

Deibner *et al.* (1963) studied the lead acetate precipitation methods using red wines and solutions of pure malvin, peonin and oenin. They found that the addition of basic lead acetate alone or with subsequent pH adjustment was insufficient to precipitate the Acys completely. The adjustment of the pH with ammonia to 10.2–10.4 prior to the addition of lead acetate, resulted in an Acy-free supernatant which was considered as a sign of complete precipitation. These authors also evaluated the Official German Method for the detection of hybrid character in wines employing fractional precipitation with neutral lead acetate followed by pH adjustment. Their results showed that neither the fractionation nor the precipitation was complete. The authors indicated that not only quantitative but qualitative changes in Acy composition can occur as the result of lead acetate precipitation.

Polyamide column chromatography

In comparison with other flavonoids, relatively little use has been made of this new adsorbent in Acy analysis. Chandler *et al.* (1959) found that Acys and anthocyanidins were only slightly retained on polyamide columns, being readily eluted by the usual Acy solvents with sufficient acid to prevent fading of the pigments. The Acys were successfully separated from the other flavonoid compounds which were firmly retained and from polymeric material which was completely retained. Polyamide has been used for the preliminary purification of blackcurrant (Chandler *et al.*, 1962) and cranberry (Zapsalis *et al.*, 1965) Acys. Gallop (1965) found polyvinylpyrrolidone preferable to polyamide for the purification of Acy solutions. Polyvinylpyrrolidone was also used with Acys by Blundstone *et al.* (1966).

Ion exchange resin column chromatography

According to Seikel (1962) it was Ice *et al.* (1951) who first suggested the use of ion exchange resins (Amberlite CG-50) for the preliminary purification of Acys. All flavonoids except chalcones (Bate-Smith *et al.*, 1953) are absorbed from aqueous solutions by the resin (Amberlite IRC-50). Most of the water-soluble organic and inorganic impurities can be removed by exhaustive washing with water. Glycosides can be eluted with aqueous alcohol and the aglycons with 95% alcohol.

The mechanism of adsorption is not clearly understood. Boardman (1957) indicated that the various types of cation exchange resins behave differently. Ionic aromatic substances may be strongly adsorbed on polystyrene but may show lower adsorption on ionized polymethacrylic acid resins or on cellulose ion exchangers. The carboxylic groups on polyacrylic acid resins will be unchanged below pH 5.5 and may bind polar substances by polar binding. This is really adsorption chromatography on ion exchange resins.

Various type of resins (Amberlite CG-50, IRC-50, IR-120, Dowex 50, 50W and Zeocarb 215) have been used for preliminary purification of Acys, but there is very little information on the quantitative recovery of Acys from ion exchange columns. According to Kazmierczak (1966) the Acys from various berry juices were completely absorbed on Amberlite IRC-50, IR-120 and Dowex 50 columns. The Acy was readily eluted with 95% ethanol from the Amberlite IRC-50 column, but only with difficulty from the other two resins. However, some authors (Parkinson, 1954; Endo, 1958; Sakamura *et al.*, 1961; Gromeck *et al.*, 1964) noted that a colored (usually orange or brownish) effluent occurred upon washing the Acys absorbed on the column with water.

Methods other than those evaluated here, such as mercury and zinc acetate precipitation (Biol *et al.*, 1963), solvent extraction (Nebesky *et al.*, 1949), cellulose column chromatography (Daravingas *et al.*, 1965), electrophoresis (Markakis, 1960) and thin-layer chromatography were or could also be used for the preliminary purification of Acys.

MATERIAL AND METHODS

The "Ocean Spray" brand CrJ used was obtained in 1-pint bottles from the manufacturer (Ocean Spray Cranberries Inc., Hanson, Mass.)

Unsweetened Cr juice (Cr juice in the following) was obtained by pressing the thawed berries on a Carver Model K Laboratory Press (F. S. Carver Inc., Summit, N.J.) at 10,000 psi pressure. The cranberries (Cr) used (*Vaccinium macrocarpon* Ait., var. Howes) were grown at the University of Massachusetts Agricultural Experiment Station, Wareham, Mass., and stored at 0°F until used.

Evaluation of the total anthocyanin recovery

The purification treatment was carried out with accurately measured volumes of CrJ as described later. The T Acy was determined on the following fractions:

1. Untreated CrJ.
2. Aqueous eluate or filtrate containing sugar and some Acy.
3. Partially purified Acys (Crude Acys).

The T Acy determination was carried out on the CrJ and on the aqueous fraction using either the pH differential or the single pH method developed for CrJ (Fuleki *et al.*, 1968b). In some cases, the Degradation Index (DI) was also calculated (Fuleki *et al.*, 1968b). The T Acy in the Crude Acy was determined using the method developed for Cr extract (Fuleki *et al.*, 1968a).

Evaluation of the recovery of individual anthocyanins

Since the CrJ could not be chromatographed directly, the comparison on I Acy composition between treated and untreated samples was made using Cr juice. Both the Crude Acy and the untreated Cr juice were chromatographed and the percent composition of individual Acys determined (Fuleki *et al.*, 1968c). The pigment composition of the purified juice was compared to that of the original using the "t test" for the statistical analysis of the data.

Precipitation with lead acetate

Basic lead acetate. A saturated aqueous solution of pH 8.4 was used. Since basic lead acetate was employed in most of the experiments, the following text will refer to this reagent as lead acetate except where neutral lead acetate was specified.

Neutral lead acetate. A saturated aqueous solution of pH 5.7 was used.

5% HCl in methanol. Conc. HCl-methanol (15:85).

Procedure for lead acetate. The procedure developed for the purification of Cr Acys was as follows: A sufficient amount of lead acetate solution was added dropwise, with vigorous stirring, to the CrJ or Cr juice, to precipitate the Acy. The juice turned to a green slurry when the reaction was complete. The pH was adjusted to 7.0–7.1 with the dropwise addition of approximately 0.5 *N* NaOH, accompanied with vigorous stirring of the slurry with a magnetic stirrer. The precipitate was filtered with suction on a medium porosity fritted glass funnel with a thin layer of filter aid (Hyflow Supercel, Johns-Manville Corp., New York, N. Y.). The precipitate was washed with distilled water and finally with acetone.

The loss of Acy in the filtrate was detected by adding a few drops of approximately 2 *N* HCl to the bottom of the beaker in which the filtrate was collected before starting the filtration. The appearance of a pink color indicated that Acys were present in the filtrate. The precipitate was transferred to a centrifuge tube, in which the Acys reconverted with 5% HCl in methanol, and the supernatant containing the Crude Acys was separated from the $PbCl_2$ crystals by centrifuging.

Difficulty was experienced in measuring the pH of the precipitate. The sticky precipitate poisoned the electrodes rapidly so the response became very sluggish. Indicators could not be used because the dyes reacted with the free lead acetate. To prevent poisoning, the electrodes were washed after every measurement with approximately 2 *N* HCl, followed by generous rinsing with distilled water. The electrodes were stored overnight in the HCl solution in order to dissolve any adsorbed lead salt.

Polyamide column chromatography

Procedure for polyamide. The preparation of Ultramid, B.M. (Badische Anilin und Soda Fabrik, Ludwigshafen, Germany) polyamide powder for use as absorbent was essentially the same as described by Chandler *et al.* (1962). The prepared absorbent was slurried into all glass chromatography columns (300 × 10 or 400 × 20 mm) and washed with water. The CrJ or juice was absorbed on the top of the column and the sugar was eluted with water. The Acy was eluted from the polyamide with either ethanol—0.5 *N* HCl (1:1) or the upper layer of 1-butanol-*N* HCl (1:1). Although the latter eluant gave a more compact band, the ethanolic eluant was used more frequently because the resulting ethanolic Crude Acy solution was handled more conveniently in subsequent analysis.

Polyvinylpyrrolidone (Polyclar AT; General Aniline and Film Corp., New York, N. Y.) columns were prepared as described by Gallop (1965). The CrJ was absorbed on the column and the sugar was washed down with 0.25 *N* HCl. The Acy was eluted with 0.1% HCl in 80% methanol (Blundstone *et al.*, 1966).

Evaluation of total anthocyanin recovery. The general procedure was followed with a few modifications. Large size columns (400 × 20 mm) packed about ⅔ full with polyamide were used. The O.D. of the collected fractions diluted with the pH 1.0 and 4.5 buffers was measured at the wavelength of maximum absorption, namely at 510 nm for Cr and cherry and 500 nm for strawberry.

Ion exchange resin column chromatography

Selection of the ion exchange resin. A simple batch process was used for the screening of the following resins: Amberlite CG-50 and IR-4B (Rohm & Haas Co., Phila., Pa.), Rexyn 102 (Fisher Sci. Co., Fair Lawn, N. Y.), Bio-Rad AG-50 (Bio-Rad Laboratories, Richmond, Calif.), Zeo-Karb, Permutit Q and H-70 (Permutit Co., New York, N. Y.).

Procedure for anthocyanin purification. The Amberlite CG-50, a weakly acidic cation exchange resin of 100–200 mesh, was hydrated by soaking the resin in water and occasionally decanting the silky supernatant in order to remove the undersize particles. All glass 300 × 10 mm chromatography columns equipped with fritted glass filters were used. To prevent small resin particles from passing through the filter, a polyvinyl chloride (Polyvic) filter disc (Millipore Filter Corp., Bedford, Mass.) was fitted on top of the glass filter. A sufficient amount of resin slurry was poured into the glass column to form a 15–18 cm

column after the resin settled. Ten ml CrJ or 5 ml Cr juice were adsorbed on the drained column. The sugar was washed down with 7 ml of distilled water and Acys were eluted with 20–25 ml of 0.25% conc. HCl in methanol which was added to the column in small increments. The dense part of the Acy band eluted with methanol was collected (Crude Acys) for the determination of the ratio of individual Acys by chromatographic analysis (Fuleki *et al.*, 1968c).

Establishment of the elution profile. The standard procedure was used. The only exception was that the aqueous as well as the methanolic eluates were collected as small fractions. To follow the elution of sugar, the concentration of soluble solids in the aqueous fractions was measured with an Abbe-type refractometer. The aqueous fractions were diluted with pH 1.0 buffer, while the methanolic eluate was diluted with EtOH-1.5*N* HCl (85:15) and the T Acy content was measured using the single pH methods.

Although degraded pigment occurred in the aqueous fractions, the pH differential method could not be applied because of the small volume of the fractions. To account for the degraded pigment, a correction factor was established by collecting the aqueous eluate from 5 columns run at the same time and from the same original sample as that used for the profile analysis. The Acy content from the 5 eluates was determined using the pH differential method.

Using the same data, but considering only the O.D. reading obtained at pH 1.0, the Acy content was calculated using the single pH method. The pH differential method gave 0.174 mg Acy, whereas the single pH method indicated 0.277 mg Acy content for the aqueous eluate originating from 50 ml CrJ. It was assumed that the same relationship would apply for the aliquots obtained for the elution profile; therefore a correction factor could be established the same way as the DI was determined by dividing the result of the single pH method with that of the pH differential method, namely 0.277/0.174 = 1.59. Consequently the Acy readings in the aqueous fractions obtained for the elution profile using the single pH method were corrected by dividing them by 1.59.

RESULTS AND DISCUSSION

Precipitation with lead acetate

Establishment of operating parameters. Neutral lead acetate was first used but it did not precipitate the Acys completely and also the proportions of the I Acys were changed considerably. Chromatographic analysis of the fractions precipitated with neutral and subsequently with lead acetate revealed that the fraction precipitated with neutral lead acetate was enriched in cyanins while the proportion of peonidin glycosides increased in the supernatant. This showed that neutral lead acetate was unsuitable for quantitative purposes and further work was carried out using basic lead acetate as precipitant.

To determine the optimum pH, 2 ml of lead acetate were added to 25-ml aliquots of CrJ and adjusted with 0.5 *N* NaOH to various pH levels. The standard procedure was followed except that an aqueous, instead of a methanolic, solution of HCl was used for recovery.

The results in Table 1 indicated that the optimum pH occurred at or slightly above 7.0. The T Acy recovered in the filtrate was measured in some cases and is presented in parentheses after the percent T Acy recovery in the major fraction. By subtracting the sum of the recoveries in the two fractions from 100, the percent loss was obtained.

Since the reconversion of Acy was carried out in an aqueous medium and the thoroughly washed lead chloride crystals were perfectly white, the Acy loss could not be explained by irreversible absorption of Acy on the $PbCl_2$ crystals. Since the loss increased considerably over pH 10.0, possibly the loss was due to alkaline destruction of the Acy. Also, in the pH range between 6.9 and 10.0 the filtrate did not appear to contain any Acy, but the loss of Acy was considerable (10.4% at pH 9.7). This shows the limited value of a negative test on the filtrate or supernatant which is frequently claimed to be the sign of a complete recovery (Deibner *et al.*, 1963).

The addition of lead acetate neutralized the acids and this increased the pH of the CrJ considerably (2 ml lead acetate added to 25 ml CrJ increased the pH from 2.8 to 5.9). This increase of pH was not high enough to insure complete precipitation of the Acys; therefore addition of dilute alkali was necessary. The order of addition of the lead acetate (before or after the pH adjustment) had a definite influence on the reaction. Higher recoveries (Table 1) were obtained by adjusting the pH after lead acetate addition. Consequently, this order of addition was adopted for the standard procedure.

It was difficult to specify the exact amount of lead acetate which should be added to a 25 ml CrJ sample. The lead ion reacts not only with the compounds present in the CrJ but also has a tendency to polymerize as the pH increases (Mattock, 1954). To avoid this phenomenon, which apparently occurred as a local excess built up, the CrJ was stirred vigorously with a magnetic stirrer, while adding the lead acetate. Under such conditions, 2 ml of saturated lead acetate solution were more than sufficient to precipitate all the Acy in a 25-ml CrJ sample, as indicated by the color of the resultant slurry.

Filtration of the precipitate, instead of the customary centrifuging, was found to be a better method. The sugar could be completely removed by washing the precipitate on the filter and the filtrate also appeared clearer than the supernatant. To prevent clogging, a thin layer of filter-aid was deposited on the fritted glass filter of the Hirsch type funnel.

Some Acy was retained on the lead chloride crystals when the reconversion was carried out in an alcoholic medium. An effort was made to correct for this by replacing the hydrochloric acid with sulfuric acid. However, more Acy was absorbed on the lead sulfate than on the chloride with both alcoholic and aqueous media. Lead chloride is fairly soluble and the sulfate is barely soluble in water. This suggests that the $PbCl_2$ crystals appeared free of Acy after washing with aqueous HCl, because the water dissolved the surface layer with the absorbed Acy.

The crystals appeared pink in all other cases, because of the low solubility of $PbCl_2$ in methanolic and $PbSO_4$ in both media. Since it was desirable to recover the Crude

Table 1. Recovery of total anthocyanin after basic lead acetate purification of cranberry juice cocktail at different pH levels.

Treatment	Experiment No. 1	2	3	4	5
			mg/100 ml		
T Acy [1]	5.05	5.86	5.96	4.81	4.76
			ml		
CrJ	10.0	20.0	20.0	25.0	25.0
Lead acetate	0.25	1.0	1.5	2.0	2.0
Adjusted pH [2]		Recovery % [3]			
5.9		89.0(9.0)			
6.0			85.0(+)		
6.2					89.3(3.8++)
6.5		89.0(4.0)	98.0(+)		90.7(1.8+)
6.8		95.0(+)			
6.9		99.3(−)			93.3(1.1−)
7.0			99.5(−)		92.5(0.6−)
7.1					95.6(−)
7.2	86.0(++)	88.0(3.0)			
7.3	86.6(++)			95.7(−)	95.4(−)
7.4	86.6(++)	90.0(2.0)		97.9(−)	
7.5			94.0(−)	93.7(−)	
7.7					91.5(−)
8.0			97.5(−)		
8.7					91.5(−)
9.7					88.6(1.0−)
10.9					68.2(8.3++)
11.2					53.1(15.0++)

[1] The T Acy determinations were carried out using the pH differential method in No. 1, 4, 5 and the single pH method in No. 2, 3 experiments.

[2] The results are underlined in cases where the dilute alkali used for the pH adjustment was added prior to the addition of lead acetate. The pH values listed give the final pH of the slurry measured after the addition of dilute alkaline and/or lead acetate.

[3] The values in parentheses give the Acy % recovery in the filtrate. The results of the visual estimation of Acy content are given as follows: + = trace; ++ = fair amount; − = none.

Acy in an alcoholic solution for chromatographic analysis, the methanolic medium was retained for the reconversion of Acy from the precipitate. The lead chloride crystals were separated from the Acy by centrifuging, because some lead chloride passed through even the fine porosity glass filter.

Total anthocyanin recovery. The results in Table 1 show that excellent recoveries could be obtained with lead acetate following the developed procedure but some unexplainable variation also occurred.

Recovery of individual anthocyanins. Data for 15 untreated and 6 samples of each of the treatments are presented in Table 2. There was no significant difference at the 5% level for any of the major Cr Acys, and the agreement between the treated and untreated samples was good.

Since methanol was selected as medium for the reconversion of Acy, it was important to determine whether the loss due to irreversible absorption on the lead chloride crystals occurred at the same rate for all four pigments. To explore this question, the precipitate was divided and one part of it was reconverted in an aqueous and the other in the standard methanolic medium. The excellent agreement between the two treatments (Table 2) indicated that it was quite safe to use methanolic HCl for the reconversion of Acy.

Purification profile

Filtrate. It contained practically all the sugars; therefore, the method effectively purified the Acys of CrJ for chromatographic analysis. If the conditions were not controlled carefully, some Acy was lost in this fraction.

Crude Acy. The fraction obtained by reconverting the precipitate contained not only the Acys but a large number of other compounds present in the CrJ as well. The presence of degraded Acy in this fraction can be quite troublesome, particularly when the determinations are carried out on old samples containing a large proportion of degraded pigment. The DI was calculated for this fraction in cases where the pH differential method was used, and it was found to be the same as that determined for the untreated CrJ sample. This and chromatographic analysis of the Crude Acy showed the presence of degraded pigments in this fraction.

Anthocyanin retained on the lead chloride. When an alcoholic medium was employed for recovery, some Acy was irreversibly absorbed on the crystals, but the I Acy composition did not change as the result of this loss.

Degraded Acy. If any anthocyanidins were present in the samples, they would be degraded in the alkaline medium. Some anthocyanin may have been lost due to alkaline degradation.

Table 2. Effect of lead acetate treatment on the individual anthocyanin composition of cranberry juice.

		Lead acetate treated[3]				
	Untreated[2]	Reconverted in MeOH		Reconverted in water		
Pigment[1]	%	%	p	%	p	p[4]
Pn-3-Ar	18.8 ± 0.37	18.2 ± 0.65	0.42	17.9 ± 0.28	>0.05	>0.62
Cy-3-Ar	19.2 ± 0.48	18.0 ± 0.52	0.09	18.4 ± 0.51	0.16	>0.48
Pn-3-Ga	38.3 ± 0.47	39.0 ± 0.67	>0.37	39.5 ± 0.69	>0.13	>0.55
Cy-3-Ga	23.7 ± 0.51	24.8 ± 0.78	>0.23	24.2 ± 0.38	>0.42	0.42

[1] Cy = cyanidin, Pn = peonidin, Ga = galactoside, Ar = arabinoside.
[2] Mean values of 15 identical samples with the standard deviation of the mean.
[3] Mean values of 6 identical samples for each treatment with the standard deviation of the mean.
[4] Probability of observing a greater difference between duplicate samples than that found between the methanol and water media used in the reconversion of anthocyanins.

Polyamide column chromatography

Evaluation of total anthocyanin recovery. Fairly good Acy recoveries of Acys could be obtained with Cr juice (Table 3). The major shortcoming of the polyamide treatment was that the Acy diffused on the column. The result of this was a rather dilute Crude Acy solution. An additional disadvantage of this absorbent was that the elution was slow. Purification on polyvinylpyrrolidone column was also slow and the purified Acy solution was dilute.

The general profile of Acy elution from polyamide columns is shown in Table 3. Some pigment was lost for chromatography, as with the ion exchange treatment in the aqueous washings. However, the pattern of Acy loss was different. Visual observation revealed that the Acy loss did not occur as a peak but at a steady rate and it continued as long as the column was washed with water. The low value of the DI indicated that most of the Acy in the aqueous fraction was non-degraded pigment. The DI for the other fractions showed that while the first ethanolic fraction was almost completely free of degraded pigment, the second one contained a large proportion. Visual examination of the eluted column showed that a reddish-brown fraction, which was apparently degraded Acy, was retained in diffused form on the column.

Chandler *et al.* (1959) indicated that a firmly absorbed polymeric material would prevent the repeated use of this absorbent. The polyamide treatment gave similar elution profiles with CrJ, strawberry and sweet cherry juices. Since polyamide treatment produced a very dilute purified Acy solution, which was unsuitable for chromatography, use of this absorbent was discontinued.

Ion exchange resin column chromatography

Establishment of operating parameters. Amberlite CG-50 and Rexyn 102 were the only resins among those tried which permitted the complete desorption of Acy when eluted with 95% EtOH (Fuleki, 1967). Since the Amberlite CG-50 resin showed somewhat better performance than the Rexyn 102, this resin was selected for further experimentation. The sample applied on the column had to be large enough to give about 1 to 2 ml Crude Acy solution. Ten ml fresh CrJ or 5 ml fresh Cr juice were adequate and a 1 × 15 cm hydrated resin column gave ample capacity to absorb the Acy present in the sample. To elute the sugar, it was necessary to wash the column with a minimum of 7 ml water after the CrJ was absorbed on the column.

Table 3. Recovery of total anthocyanin after polyamide purification of cranberry juice.

Fraction	ml	T Acy mg	Recovery %	Degradation index
Cranberry	300			
Aqueous eluate	243	0.33	1.9	1.09
Ethanolic eluate (1)	176	15.63	89.0	1.04
Ethanolic eluate (2)	150	0.28	1.6	2.11
Total	569	16.24	92.5	
Original juice	300	17.56	100.0	1.20

The behavior of the Acys on the ion exchange resin indicated that these pigments were absorbed on the resin primarily by non-ionic forces. The effective pH range of the resin was pH 5 to 14, while the pH of the CrJ was 2.8. Furthermore, the resin also absorbs flavonoids (Seikel, 1962) which, unlike the Acys, lack the positive charged form (oxonium ion) that could be responsible for the ionic absorption on a cation exchange resin. It appears that hydrogen bonding and van der Waals forces rather than ion exchange reactions are involved in the absorption of these substances on Amberlite CG-50 resin. It is probable that the unusual macroreticular physical structure of the Amberlite CG-50 resin (Kunin, 1964) was partially responsible for its favorable performance characteristics.

The absorption took place from an aqueous medium and the Acy could be readily eluted from the column with ethanol, methanol and acetone in which the Acy was soluble, but not with diethylether. Elution with acetone was too slow. Water in the ethanol caused tailing. Alcohol without acid caused a red to violet color change on the column, indicating the possibility of degradation via the quinoidal form. Based on the above considerations, 0.25% conc. HCl in anhydrous methanol was selected as the eluting solvent. The addition of the eluting solvent in small increments, particularly at the beginning of the elution helped to reduce the diffusion of the band. Approximately 25 ml of solvent were required to elute the Acy from the column.

Elution profile. To evaluate the effectiveness of the developed procedure, an elution profile was established by measuring the soluble solids and Acy contents of successive aliquots eluted from the column (Fig. 1). The procedure

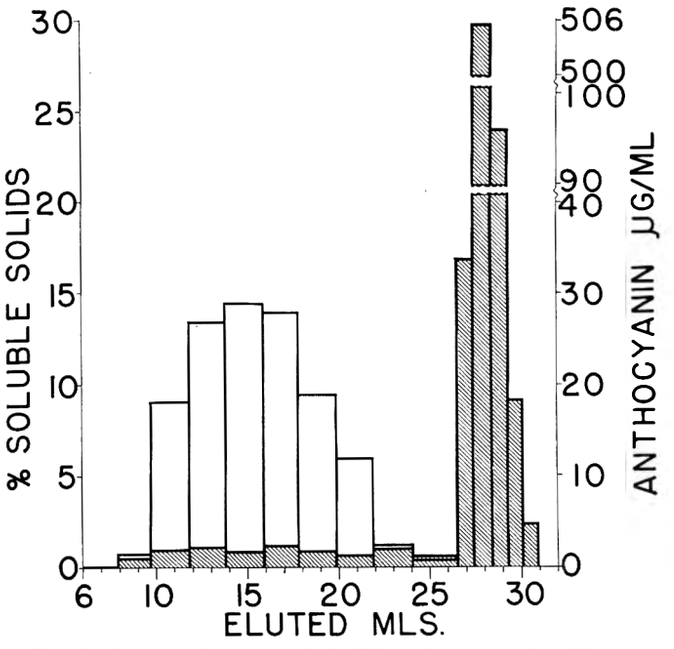

Fig. 1. Elution of soluble solids (□) and anthocyanins (■) present in cranberry juice cocktail from a 15.5 × 1.0 cm Amberlite CG-50 ion exchange resin column. (The methanolic eluant was added after collecting 15.9 ml eluate. The first fraction in which methanol was detected occurred after 26.5 ml was eluted.)

effectively separated the Acy from the sugar. The Acy was eluted from the column as a narrow band in front of the methanolic phase as the water was replaced from the column by the alcohol. The treatment gave a highly concentrated methanolic Acy solution.

The elution profile shows that a small amount of Acy occurred in the aqueous fractions. It was interesting to note that a small peak in Acy content occurred in the same aliquots which had the highest concentration of soluble solids. On a few columns, a larger volume of water (20 ml) was used for the elution of sugars. In these cases, no Acy could be detected in the last 5 ml of the aqueous phase.

A third colored fraction occurred when old CrJ or juice was applied on the column. The highly polymerized Acy degradation products which were apparently insoluble in water or alcohol remained on the top of the column and appeared in the form of a reddish-brown band. This demonstrated that the ion exchange treatment purified the Acys not only from the sugars but from the highly polymerized degradation products as well.

Total anthocyanin recovery. Further information was obtained on the loss of Acy in the aqueous fraction by determining the T Acy recovery in the aqueous and methanolic eluate using the pH differential method. The mean of the results obtained on triplicate samples of CrJ showed that 12.4 and 89.0% of the T Acy was recovered in the aqueous and methanolic eluate respectively. This indicated that no Acy was retained on the column.

Kazmierczak (1966) claimed that the Acys from various berry juices were completely absorbed on the Amberlite IRC-50 (chemically similar to CG-50) ion exchange column while the other juice components including the red-brown Acy decomposition products passed through the column. The chromatographically pure Acy could be readily eluted with 95% ethanol. The above author, in cooperation with other researchers (Lempka *et al.*, 1966), developed a T Acy determination method for stored juices based on the removal of Acy degradation products on an ion exchange column, prior to the absorptiometric measurement.

Contrary to Kazmierczak's findings, the results presented here show that some nondecomposed Acy was present in the aqueous eluate. This indicated that an error was introduced when only the alcohol-eluted fraction was used for T Acy determination. The fact that a larger than the original proportion of the water-soluble Acy decomposition products was present in the aqueous eluate was demonstrated by comparing the DI of the aqueous and methanolic eluates. The DI was approximately 1.03 for the untreated CrJ, 1.1 for the methanolic and 1.5 for the aqueous eluates. The large difference between the methanolic and aqueous eluates showed that a considerable portion of the soluble colored decomposition products was eluted with water.

To obtain further information, the water-eluted colored fraction was passed repeatedly through freshly prepared ion exchange columns. Visual observation showed that a fraction of the Acy from the aqueous eluate was always retained on the column, but most of the colored material passed through the column. This also demonstrated that intact Acy was present in the aqueous eluate even after repeated passage through ion exchange columns. This conclusion was supported by paper chromatographic data which showed the presence of all major Cr Acys in the aqueous eluate.

It should be mentioned here that not all the juice constituents, other than the Acys, were eluted in the aqueous washings as claimed by Kazmierczak (1966). The chromatograms of ion exchange treated CrJ and juice viewed under UV light or treated with aluminium chloride consistently showed the presence of phenolic acids and flavonoids as well. This is not surprising since the same ion exchange procedure used for Acy is also used for the purification of flavonoids (Seikel, 1962).

Recovery of individual anthocyanins. The percent contribution of each Acy was averaged from 8 and 10 chromatographic applications for the untreated and ion exchange treated juice respectively. Each of the treated samples was obtained from a separate column. There was no significant difference in the individual Acy composition of treated and untreated Cr juice (Table 4). The difference was greatest for the two galactosides which

Table 4. The effect of ion exchange treatment on the individual anthocyanin composition of cranberry juice.[1]

Pigment[2]	Untreated %	Treated %	P
Pn-3-Ar	18.7 ± 0.42	18.9 ± 1.02	>0.84
Cy-3-Ar	18.2 ± 0.60	18.4 ± 0.39	>0.76
Pn-3-Ga	39.4 ± 0.50	40.5 ± 0.30	0.06
Cy-3-Ga	23.7 ± 0.29	22.2 ± 0.86	>0.09

[1] The results are based on 8 and 10 determinations carried out on the same lot of Cr juice, for the untreated and ion exchange treated samples respectively.

[2] Cy = cyanidin, Pn = peonidin, Ga = galactoside, Ar = arabinoside.

Table 5. Performance characteristics of the anthocyanin purification methods.

Performance characteristic	Basic lead acetate	Polyamide	Ion exchange
Recovery of Acy			
Total Acy	92–98%	92%	89%
Individual Acy	good	good	good
Purification profile			
Aqueous fraction	none to some	some	some
Crude Acy	concentrated	diluted	concentrated
Retained Acy	very little	some	none
Destroyed Acy	none to some	none	none
Purity of crude Acy			
Sugars	none	none	none
Degraded Acy	all	some	traces
Flavonoids	present	present	present
Phenolic acids	present	present	present

were present in the greatest quantities. Considering the limitations of the I Acy determination method (Fuleki *et al.*, 1968c) the probability of real differences between the treated and untreated samples was sufficiently low even for these pigments. On the basis of this information, the ion exchange treatment was accepted as a reliable purification method for Cr Acys.

Recommended procedure

The aim of the purification method was to obtain a concentrated alcoholic Acy solution, free of sugar and preferably of degraded pigment as well, without altering the original individual Acy composition of the sample. The methods included in the experiments (Table 5) accomplished these aims to a varying degree.

Polyamide was rejected because it gave a more dilute Acy solution than the original CrJ. Both the developed lead acetate and ion exchange method could be used for the purification of Cr Acy prior to the determination of the ratio of I Acys. These treatments effectively removed the sugar without changing the percent I Acy composition of the sample. The purified Acy was in the form of a concentrated alcoholic solution which was well suited for further analysis. Nevertheless, the ion exchange method was favored over lead acetate precipitation because of better performance characteristics in ease of handling, consistency of results and purity of the treated Acy.

The Acy purification methods recommended for CrJ can be applied for other fruit products as well. However, before either the ion exchange or the lead acetate method is used for such a purpose it is necessary to determine whether any change in the ratio of the particular combination of I Acys occurs as an undesirable side-effect of the treatment. This danger exists particularly when acylated pigments are present and the lead acetate precipitation method is used.

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S. L. CHEN and FELIKS GUTMANIS
Research Department, Universal Foods Corporation, Milwaukee, Wisconsin 53202

Auto-Oxidation of Extractable Color Pigments in Chili Pepper with Special Reference to Ethoxyquin Treatment

SUMMARY—Deterioration of extractable color pigments in dehydrated, ground chili peppers during storage was shown to be an auto-oxidative process having the kinetics of a second order reaction. Consequently, the reaction rate constant, k_s, was used to evaluate the effect of a number of variables, such as moisture content, storage atmosphere and ethoxyquin treatment. In an oxygen-containing atmosphere, the rate constants for color deterioration varied with moisture content. The k_s value was 2 to 3 times higher at 4 to 5% moisture content than at 8 to 9%. Treatment with 100 ppm ethoxyquin afforded both substantial protection against color deterioration and an improvement of the surface color of the paprika in storage. Such treatment was most effective in low-moisture chili peppers. The color stability of several varieties was compared under controlled conditions. Some varieties were found to be more stable than others.

INTRODUCTION

THE MAJOR COLOR PIGMENTS of chili pepper *(Capsicum annum)* are the carotenoids capsanthin, capsorubin, beta-carotene, zeaxanthin and cryptoxanthin (Cholnoky, 1939). Other minor pigments, such as crytocapsin, lutein epoxide, antheraxanthin, violaxanthin and mutatoxanthin, have also been identified (Cholnoky *et al.*, 1955). The pigments may be extracted from dehydrated, ground chili peppers with solvents, such as acetone, ethyl alcohol, isopropyl alcohol, and determined spectrophotometrically (Schuster *et al.*, 1954; Moster *et al.*, 1952, 1957; Anon., 1960). Although the method is not specified for individual pigments, it provides a quantitative measure of the total concentration of pigments, which reflects the quality of chili pepper. This method is readily applicable to the study of deterioration of these pigments during processing and storage. In processing, deterioration of the extractable color pigments in chili peppers is affected by variables, such as drying temperature (Lease *et al.*, 1962) and drying rate. During storage, moisture content, atmosphere and temperature are critical factors (Lease *et al.*, 1956a).

This paper reports findings on the deterioration of these pigments during storage and its curtailment by ethoxyquin treatment. The relative color stability of several chili varieties was also compared.

MATERIALS AND METHODS

Preparation of ground chili pepper

Fully-ripened chili pepper pods were harvested, cleaned and sliced into pieces about 1–2 cm in width. They were dried to 5 to 6% moisture, either in a laboratory, forced-air tray drier at a constant temperature of 65°C, or in a commercial tunnel-drier. The dehydrated pepper was then ground in a pulverizing mill to pass standard screens of different sieve openings.

Assay of extractable pigments

On a dry solids basis, 1.25 g of ground chili peppers were extracted with 25 ml of acetone in a stoppered 250-ml Erlenmeyer flask for 3.5 hr at room temperature on a Burrell wrist-action shaker. The slurry was filtered by gravity and the residue washed with acetone to obtain a final volume of 25 ml. A 1:50 dilution of the extract was used for measurement of optical absorbance at 470 mμ, against an acetone blank.

Regulation of moisture content during storage

Since moisture content affects the color stability of chili pepper, it must be controlled. This was accomplished by equilibrating the samples over different saturated salt solutions. Since the equilibrium relative humidities of these solutions (see Table 3) ranged from 32 to 75%, several moisture levels in the equilibrated peppers could be obtained.

Determination of moisture content in chili pepper

An accurately-weighed ground chili sample (approximately 2 g) was dried in a weighing pan for 18 hr in a vacuum oven (34 mm mercury) at 80°C. Moisture content was calculated from the weight loss. Results of this method are in good agreement with those of the benzene distillation method.

EXPERIMENTAL RESULTS

Kinetics of color deterioration in chili pepper

The rate of color deterioration in chili pepper is related to the concentration of extractable color pigments. The kinetics of this reaction are illustrated below with data from a typical experiment. In this case, the ground sample (Chili Variety No. 1, 40-mesh) was stored in darkness at 40°C for 12 months. The moisture content of this sample was maintained at 4.0 to 4.7% by equilibrating it over a saturated $MgCl_2$ solution. The storage chamber was opened daily for 5 min to replenish the atmospheric oxygen. The color deterioration curve for this sample is shown in Fig. 1.

The curve shows that the half-life period, $t_{1/2}$, of this reaction is far from constant. For example, the first half-life period (from E_{470} = 0.500 to 0.250) can be estimated from this graph as only 1.4 months. But, the second half-life period (from E_{470} = 0.250 to 0.125) increases to about 3.0 months (1.4 to 4.4 months), and the third half-life period further increases to 5.5 months. When the Log E_{470} values are plotted against storage time, a non-linear relationship is obtained. This indicates that the color deterioration process of chili peppers is not a simple first order reaction.

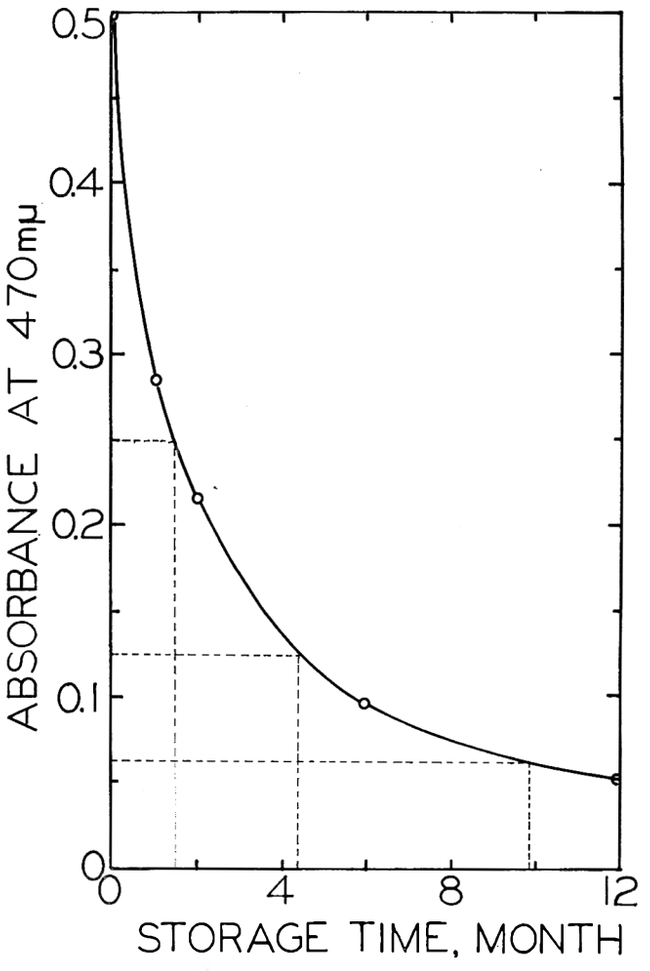

Fig. 1. Color deterioration curve of chili pepper during storage: extractable color content vs. storage time. Successive half-life periods, $t_{1/2}$, are estimated from this graph to be 1.4, 3.0 and 5.5 months, respectively. (See text).

This same set of data was used for plotting the reciprocal of color pigment concentration ($1/E_{470}$) vs. storage time, as shown in Fig. 2. In this case, a straight line is obtained, showing that the over-all kinetics of color deterioration follow the course of a second-order reaction. The calculated reaction rate constants, k_2, for the 12-month storage period are shown in Table 1.

It is apparent that these k_2 values are reasonably constant (range from 1.31 to 1.51) throughout the entire 12-month storage period. It is also shown in Table 2 that the observed half-life periods, as estimated from the color deterioration curve in Fig. 1, are equal to the reciprocals of the respective $k_2.a$ value, where a is the initial color pigment concentration. All these results prove that the rate of color deterioration in chili pepper is proportional to the square of the existing color pigment concentration, as follows:

$$\frac{dx}{dt} = k_2(a - x)^2$$

Therefore, the second-order reaction rate constant, k_2, should be used to evaluate the effect of different factors on color deterioration or the color stability of various chili samples.

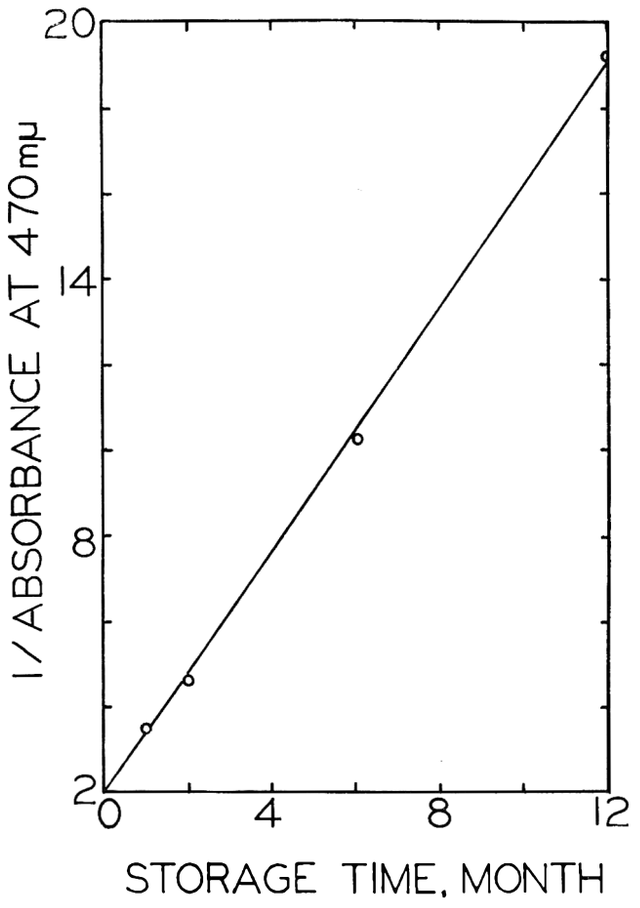

Fig. 2. A second-order reaction plot of color deterioration in chili pepper: reciprocal of extractable color content vs. storage time.

Effect of moisture content on color deterioration

The rate of color deterioration in chili pepper is affected by its moisture content. For this reason, the moisture level of the peppers must be held constant during storage. This was accomplished by equilibrating the samples, during storage, over various saturated salt solutions, such as $MgCl_2$, K_2CO_3, NaBr, Na-acetate and NaCl. The equilibrium relative humidities of these solutions range from 32 to 75% (Wink and Sears, 1950), giving equilibrium moistures in chili samples of 4 to 24% (Table 3).

In an experiment investigating the effect of moisture content, chili samples of different moisture contents were stored at 40°C for 12 months in darkness. Air atmosphere was replenished daily. The concentration of extractable color pigments was determined after 1, 2, 6 and 12 months. The second-order reaction rate constants were calculated from these determinations (see footnote to Table 1). Data are shown in Table 4.

In the case of Variety No. 1, the color deterioration rate constant, k_2, for the 4 to 5%-moisture sample (i.e. over $MgCl_2$ solution) was about twice that for a 7.5 to 9.3% sample (i.e. over K_2CO_3 solution). For Variety No. 2, the corresponding difference for the k_2 values between moisture levels was three-fold. Moisture levels higher than 8 to 9% showed no further advantage from a color stability standpoint.

Effect of storage atmosphere

Although higher moisture contents retarded color deterioration in an air atmosphere, they had no effect in

Table 1. Calculation of second-order reaction rate constants for color deterioration in chili pepper.

Storage time, t (month)	Extractable color content ($E_{470\ m\mu}$)	Reciprocal of extractable color content ($1/E_{470\ m\mu}$)	Second order reaction rate constant[1] (k_2)
0	0.500	2.00	.....
1	0.285	3.51	1.51
2	0.217	4.61	1.31
6	0.097	10.30	1.38
12	0.052	19.20	1.43
Average			1.41

[1] Rate constant, $k_2 = \frac{1}{t} \cdot \frac{x}{a(a-x)}$.
where, a = initial extractable color content;
$(a - x)$ = existing color content at time t;
x = decrease in color content during time t;
t = storage time in month.

Table 2. Calculation of half-life period, $t_{1/2}$, from the second-order reaction rate constant, k_2.

Starting extractable color content,[1] (E_{470})	Observed[1] half-life period, $t_{1/2}$	$(k_2 \cdot a)^2$	Calculated half-life period, $(1/k_2 \cdot a)^3$
0.500	1.4	0.705	1.42
0.250	3.0	0.352	2.84
0.125	5.5	0.175	5.67
0.0625	....	.....	.....

[1] Estimated from Fig. 1.
[2] $k_2 = 1.41$, the average second-order reaction rate constant (Table 1).
[3] For a second-order reaction, where $\frac{dx}{dt} = k_2 (a - x)^2$ we have, upon integrating this equation, $\frac{1}{(a-x)} = k_2 \cdot t + \frac{1}{a}$. Thus, a straight line is obtained, when the reciprocal of color content (i.e. $\frac{1}{(a-x)}$) is plotted against the storage time, t (Fig. 2). At the half-life point, where x is equal to $(a/2)$, this equation may be written as:

$$\frac{1}{(a - \frac{a}{2})} = k_2 \cdot t + \frac{1}{a} \quad \text{or,}$$

$$t_{1/2} = \frac{1}{k_2 \cdot a}$$

Table 3. Moisture content of chili pepper in equilibrium with different saturated salt solutions at 40°C.

Salt solution	Equilibrium relative humidity (%)	Equilibrium moisture content of chili pepper[1]	
		Variety No. 1 (%)	Variety No. 2 (%)
$MgCl_2$	31.7	4.5	5.5
K_2CO_3	43.1	9.3	10.6
NaBr	52.8	10.2	11.9
Na-acetate	66.7	12.1	13.5
NaCl	75.0	23.3	24.4

[1] After one-month storage.

Table 4. Effect of moisture content on color deterioration in chili pepper.

Pepper variety[1]	Moisture content (%)	Second-order reaction rate constant for color deterioration[2] (k_2)
No. 1	3.9– 4.7	1.430
	7.5– 9.3	0.682
	9.1–10.2	0.585
	12.0–12.1	0.735
	23.3–24.4	0.590
No. 2	4.5– 5.5	0.769
	8.0–10.6	0.245
	9.2–11.9	0.301
	13.1–13.5	0.178
	23.8–24.4	0.178

[1] Initial color content: Variety No. 1, $E_{470} = .500$; Variety No. 2, $E_{470} = .845$.
[2] Calculated from 12-month storage data, except for 23 to 24% moisture range (2-month storage data).

Table 5. Effect of moisture content on color stability under different storage atmospheres.

Storage atmosphere[1]	Moisture content: Initial (%)	Moisture content: Final (%)	Residual color content[2] (E_{470})
Oxygen	5.1	4.9	0.256
Nitrogen	5.1	5.1	0.560
Oxygen	9.8	9.5	0.386
Nitrogen	9.8	9.6	0.551
Oxygen	13.1	12.8	0.326
Nitrogen	13.1	12.4	0.549

[1] After equilibrating to different moisture levels, all samples (Variety No. 2) were stored at room temperature under diffused light for 95 days.
[2] The initial extractable color content was 0.650.

nitrogen atmosphere (Table 5). Therefore, the protective effect of higher moisture contents must be indirect, e.g., impedes oxygen penetration, or accelerates destruction of free radicals (O'Meara and Shaw, 1957), etc.

Color pigments deteriorated much faster in an oxygen-containing atmosphere, irrespective of other storage conditions. This is shown by data in Table 6, which is self-explanatory. It was also shown that, in air atmosphere, autoclaved chili samples were no more stable than their respective controls (Table 7). All these data show that color deterioration in chili pepper is due to spontaneous auto-oxidation, not enzyme-catalyzed oxidation, of its color pigments.

Effect of ethoxyquin treatment

Since color deterioration was oxygen-dependent, the effect of antioxidant treatment was investigated. Several anti-oxidants were evaluated. Ethoxyquin, diphenyl-p-phenylene-diamine (DPPD) were found to be quite effective. Butylated hydroxyanisole (BHA), trihydroxy-butyrophenone (THBP) were less effective; nordihydro-guaiaretic acid (NDGA) and tocopherols were ineffective. For ethoxyquin treatment, a calculated amount of ethoxy-quin/benzene solution was sprayed onto the ground chili peppers in a rotating drum. The solvent was evapo-rated and the treated samples were equilibrated to specified moisture levels with various saturated solutions. They were then stored in darkness at 40°C for 12 months (air atmosphere replenished daily). Residual color contents were determined at 1-, 2-, 6-, and 12-month intervals. Second-order reaction rate constants were calculated as previously described.

Comparative data in Table 8 show that the rate con-stants, k_2, progressively decrease with increasing ethoxy-quin levels. Therefore, the protective effect of ethoxyquin is proportional to its concentration. However, ethoxyquin treatment is more effective in low-moisture chili peppers. For example, in Variety No. 1 (4 to 5% moisture), the k_2 for the untreated control is 1.43, but the k_2 for the treated sample (1,000 ppm of ethoxyquin) is only 0.187, about 13% of the control value. After 12-month storage,

Table 7. Color stability of autoclaved chili pepper (Variety No. 1).

Treatment[1]	Moisture content (%)	Residual color content[2] (E_{470})
None (control)	4.9	.125
Autoclaved	4.6	.055
None (control)	7.1	.207
Autoclaved	7.1	.150
None (control)	9.1	.198
Autoclaved	8.5	.174

[1] Autoclaved for 15 min at 15 lb pressure. After being equili-brated to different moisture levels, all samples were stored at 40°C in darkness under a daily replenished air atmosphere for 90 days.
[2] The initial extractable color content for the first experiment was 0.452 and that for the second and third experiment, 0.400.

Table 6. Effect of storage atmosphere on color deterioration of chili pepper.

Storage time (day)	Storage atmosphere[1]	Storage temp. (°C)	Light	Moisture content (%)	Residual color content[2] (E_{470})
220	Oxygen	40	Darkness	5.1	.018
	Air	40	Darkness	4.8	.032
	Nitrogen	40	Darkness	5.3	.771
72	Oxygen	25	Darkness	11.0	.505
	Nitrogen	25	Darkness	10.0	.740
72	Oxygen	25	Diffused	10.1	.405
	Nitrogen	25	Diffused	10.2	.708

[1] 20 g of ground chili pepper (Variety No. 2) was stored in a 125-ml Erlenmeyer flask, which was fitted with a rubber stopper with two gas-exchange outlets. The flask was flushed with either oxygen, air or nitrogen for a few minutes before the outlets were closed with screw clamps.
[2] The initial extractable color content for the first experiment was 0.845 and that for the second and third experiment was 0.800.

Table 8. Effect of ethoxyquin treatment on color deterioration in chili pepper with different moisture contents.

Pepper variety	Ethoxyquin treatment (ppm)	Moisture content (%)	Second-order deterioration rate constant (k_2)	Residual color content at 12 months[1] (E_{470})
No. 1	None	3.9– 4.7	1.430	0.052
	25	4.0– 5.1	0.795	0.086
	50	4.2– 5.3	0.614	0.107
	100	4.2– 4.8	0.474	0.130
	1000	4.1– 5.0	0.187	0.235
	None	7.5– 9.3	0.682	0.098
	25	7.6– 8.2	0.755	0.091
	50	7.5– 7.9	0.589	0.110
	100	7.6– 9.4	0.334	0.165
	1000	7.2– 9.3	0.156	0.257
	None	9.1–10.2	0.585	0.112
	25	9.1– 9.7	0.590	0.110
	50	9.5–10.8	0.571	0.131
	100	9.3–10.3	0.344	0.163
	1000	9.2– 9.9	0.159	0.255
No. 2	None	4.5– 5.5	0.769	0.096
	100	4.9– 5.6	0.288	0.215
	1000	4.8– 5.2	0.132	0.360
	None	8.0–10.6	0.245	0.243
	100	8.4–10.5	0.162	0.320
	1000	8.1–10.1	0.097	0.425
	None	9.2–11.9	0.309	0.208
	100	9.4–11.7	0.154	0.331
	1000	9.1–10.8	0.097	0.425

[1]Residual extractable color content may also be calculated from the k_2 values as follows:

$$(a - x) = \frac{1}{k_2 \cdot t + \frac{1}{a}}$$

where $(a - x)$ is the residual color content at $t = 12$ months; the initial color content, a, is 0.500 for Variety No. 1 and 0.845 for Variety No. 2.

the residual color content of the treated sample is 5 times greater than that of the control (i.e. $E_{470} = 0.235$ vs. $E_{470} = 0.052$). The difference in color deterioration between treated and untreated samples diminishes with increasing moisture levels.

The protective effect of ethoxyquin on color deterioration is also reflected by the improvement of surface color of paprika during storage. For instance, in the case of paprika irradiated with ultraviolet light during storage, the surface color of an ethoxyquin-treated sample (about 100 ppm) was found more reddish than the control (Stahl, 1966). Some of these data, obtained with a Hunter Color Difference Meter, are presented in Table 9. As the exposure time increases, the surface color becomes lighter, with decreasing redness but increasing yellowness. However, in the ethoxyquin-treated sample, the ratio of redness/yellowness is higher than the control.

To appreciate the extent of visual change in the surface color of these samples, the Hunter readings, *L, a, b,* were converted, according to the established formulas (Hunter, 1958), to the C.I.E. tristimulus values, X, Y, Z. The latter values, in turn, were transformed into the more familiar Munsell notations (Newhall *et al.*, 1943). The Munsell notations (Hue, Value/Chroma) for the zero-time

Table 9. Effect of ethoxyquin treatment on surface color of paprika.[1]

Exposure time (week)	Surface color[2]							
	Untreated control				Ethoxyquin-treated sample			
	L	a	b	a/b[3]	L	a	b	a/b[3]
0	27.4	24.8	14.6	1.70	25.4	25.5	13.4	1.90
4	29.9	22.4	15.1	1.48	27.3	24.7	13.9	1.78
8	33.3	17.3	15.7	1.10	29.8	21.2	14.5	1.46
16	36.7	13.1	16.9	0.78	33.7	16.9	16.2	1.04
20	37.5	12.1	16.9	0.72	34.6	15.6	16.4	0.95

[1] Courtesy Dr. William H. Stahl, McCormick & Co., Inc.
[2] L—measures lightness and varies from 100 for perfect white to zero for black;
a—measures redness when positive;
b—measures yellowness when positive;
[3] —ratio of (redness/yellowness).

Table 10. Color deterioration in different chili pepper varieties.

Pepper variety	Moisture content (%)	Initial color content (E_{470})	Deterioration rate constant (k_2)	Residual color content at 6 months[1] (E_{470})
A	4.5– 4.7	.910	1.429	.103
B	4.2– 4.5	.720	1.844	.080
C	5.7– 6.0	.880	2.085	.073
D	4.7– 4.8	1.135	0.413	.297
E	4.1– 4.7	1.742	0.864	.173
F	4.2– 4.8	1.412	0.491	.273
G	4.1– 4.4	1.142	0.715	.193
A	6.9– 7.1	.910	0.664	.196
B	6.5– 7.0	.720	0.612	.197
C	7.8– 9.2	.880	0.694	.188
D	7.2– 7.8	1.135	0.140	.580
E	7.4– 7.9	1.742	0.415	.325
F	7.1– 8.1	1.412	0.346	.358
G	7.3– 7.7	1.142	0.408	.300
A	7.3– 8.5	.910	0.380	.295
B	7.2– 7.8	.720	0.433	.250
C	9.8–10.0	.880	0.419	.273
D	8.3– 8.8	1.135	0.091	.700
E	8.0– 8.7	1.742	0.286	.435
F	7.7– 8.4	1.412	0.230	.478
G	7.9– 8.0	1.142	0.280	.390

[1] Residual extractable color content may also be calculated from k_2 values—see footnote of Table 8.

samples were: "0.92 YR, 3.2/8.1" for the untreated, and "0.50 YR, 3.0/8.1" for the ethoxyquin-treated sample. At the end of 20 weeks, the corresponding notations were: "6.0 YR, 4.3/5.9" and "4.2 YR, 4.0/6.3," for the untreated and treated sample, respectively. These data showed that the ethoxyquin-treated sample was more reddish in hue, higher in chroma (i.e. spectrum purity) and slightly darker than the untreated control.

Color deterioration in different pepper varieties

It has been noted that the color stability in chili peppers varied from batch to batch. Some of these differences might be due to variations in processing conditions such as drying temperature, drying rate and others. Nevertheless, varietal difference could not be ignored. Therefore, the color stabilities of seven varieties were compared. Fully-ripened, shrivelled pods were harvested, cleaned, sliced and dried to 5 to 6% moisture content at 65°C, under identical conditions in a laboratory forced-air tray drier. The dehydrated chili slices were then ground and sieved through different screens, equilibrated to specified moisture contents and stored in darkness at 40°C in an air atmosphere (replenished daily) for 6 months. Aliquots of these samples were periodically removed for determinations of residual color contents. The deterioration rate constants, k_2, were calculated for comparison. The results are summarized in Table 10.

These data show that Variety D has the highest color stability, since its k_2 values are the lowest at all three moisture levels. By the same token, Variety F ranks second, followed by Varieties G and E. Varieties A, B, and C were the least stable. Such a ranking is consistent for all three moisture levels.

DISCUSSION

Data on color deterioration in chili peppers showed that the overall kinetics of the process followed the course of a second-order reaction. This finding revealed little about the mechanism of the complex reaction, but it provided a means to compare different samples and treatments.

Previous investigators used "percent of original color lost or retained" for comparing the stability of different chili samples or the effect of different treatments (Van Blaricom and Martin, 1951; Lease and Lease, 1956a, 1956b, 1962). Though not explicitly stated, they tacitly assumed the color deterioration process to be a first-order reaction. For a first-order reaction, the fractional time period (time required to decompose a definite fraction of reactant) and/or the fractional (or percent) loss of reactant per unit time would be constant. However, this is not true for the color deterioration in chili peppers, as shown by the data in Table 1 and Fig. 1. Here, the percent of original color lost during the first month of storage is 43.0% [i.e. (.500–.285)/.500].

During the second month of storage, the percent of color lost drops to 23.8%, [i.e. (.285–.218)/.285]. By the third month, the value is further reduced to 19.8% [i.e. (.215–.174)/.215]. Obviously, the percent of original color lost per month is not constant. Therefore, no single value is truly representative of the overall color stability of this particular sample. On the other hand, the second order reaction rate-constant, k_2, is constant throughout the entire storage period, regardless of whether it is calculated from 1-, 2-, 6-, or 12-month storage data (Table 1). It is, therefore, apparent that only the rate constant, k_2, not the percent of original color lost or retained, should be used in comparing the effect of different treatments or the relative color stability of different chili samples.

Although the effect of moisture content on color deterioration in chili peppers is recognized in the trade, no systematic investigation has been published. Lease and Lease (1965a) compared the color stability of different peppers with different moisture contents and different initial color contents. Due to the confounding of these three variables (pepper variety, moisture content and initial color content), the data revealed little about the effect of moisture content on color stability.

In experiments on the effect of moisture content, not only a wide range of moisture levels should be evaluated, but the storage atmosphere must be considered, since moisture effect is evident only in the presence of oxygen-containing atmosphere. No effect is observed for nitrogen atmosphere. Misleading results will be obtained in experiments where chili samples are stored in closed containers, due to gradual depletion of oxygen in air atmosphere.

Evidence has been presented to establish the auto-oxidative nature of the color-deterioration process in chili pepper. The shelf-life of various chili products may be improved by the exclusion of oxygen atmosphere. However, the incorporation of antioxidants into these products is a more practical approach. Several antioxidants have been evaluated. Their relative effectiveness is in general agreement with published findings (Van Blaricom and Martin, 1951; Lease and Lease, 1956b). The protective

effect of ethoxyquin was systematically investigated. It was found to be most effective at low-moisture contents. At 100 ppm, the maximum level approved by the Food and Drug Administration (Anon., 1965), the ethoxyquin treatment afforded both substantial protection against autooxidative deterioration of the color pigments and an improvement of the surface color of paprika. No adverse effect on flavor was noted.

A comparison of the color deterioration rate constant, k_2, for seven chili varieties showed some were more stable than others. The cause for such variation may be due to compositional differences in carotenoid mixtures, levels of naturally-occurring antioxidants, or other factors. This is currently under investigation.

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HENRY CO[a] and P. MARKAKIS
Department of Food Science, Michigan State University
East Lansing, Michigan

Flavonoid Compounds in the Strawberry Fruit

SUMMARY—The flavonoid compounds of the strawberry fruit were studied by paper chromatography, spectrophotometry and color reactions. Catechin, quercetin-3-glucoside, kaempferol-3-glucoside and leucocyanidins of varying degrees of polymerization were found besides the two anthocyanins, pelargonidin-3-glucoside and cyanidin-3-glucoside.

When the leucocyanidins were fractionated into water and methanol insoluble, ethyl acetate-soluble and water-soluble fractions, the latter fraction, generally representing glycosylated leucoanthocyanidins, was found to be the largest of the three in both ripe and unripe strawberries.

INTRODUCTION

SOME FLAVONOID COMPOUNDS of the strawberry plant have already been reported. Pelargonidin-3-glucoside and cyanidin-3-glucoside have been identified in the strawberry fruit. (Sondheimer *et al.*, 1948; Lukton *et al.*, 1955.) Kaempferol and quercetin were also identified in the fruit of the strawberry by Williams *et al.* (1952). Kaempferol, quercetin and leucoanthocyanin have been detected in the leaves of the cultivated strawberry, (Bate-Smith *et al.*, 1954) and in strawberry leaf disks maintained on sucrose solution (Creasy *et al.*, 1964).

While studying the biosynthesis of anthocyanins in the strawberry fruit (Co and Markakis, 1966), it was deemed desirable to better characterize their flavonoid constituents. The identification of some of these flavonoid compounds is reported here.

EXPERIMENTAL METHODS

Materials

Strawberry fruits of the variety Midway grown in a greenhouse were used. The unripe strawberries were picked at the green-white stage and the ripe ones when they were red and firm.

Extraction of flavonoid compounds

The strawberries were extracted four times with boiling 80% methanol. The residue was saved for the determination of the methanol and water insoluble fraction of leucoanthocyanins. The extracts were combined and the methanol was evaporated under vacuum in a rotating flash evaporator thermostated at 38°C. The residual aqueous solution was first washed five times with petroleum ether (b.p. 30 to 60) to remove chlorophyll, carotenoids and waxy material and then extracted eight times with ethyl acetate. The combined ethyl acetate extracts were dried with anhydrous sodium sulfate, filtered and concentrated to a small volume. The remaining aqueous solution was saved for the determination of the water soluble leucoanthocyanins.

[a] Present address: T. J. Lipton Company, Englewood, New Jersey.

Paper chromatography

The ethyl acetate extract was chromatographed descendingly in two dimensions on Whatman No. 1 paper using n-butanol-acetic acid-water (6:1:2 v/v/v) (BAW) as the first solvent for 15 hr and then 2% acetic acid as the second solvent for 3.5 hr.

Examination of the paper chromatograms

The air-dried chromatograms were observed under visible and UV lights, before and after fuming with ammonia vapors. UV light of two different wavelengths was used, 254 and 366 mμ. The observation under UV light was repeated after spraying the chromatogram with 5% $AlCl_3$ in ethanol.

Another chromatogram was sprayed with 0.5% $FeCl_3$ + 0.5% $K_3Fe(CN)_6$ in aqueous solution. All phenolic compounds give blue spots with this reagent. A permanent record was obtained by dipping the sprayed chromatogram in 2 *N* HCl solution followed by thorough washing in distilled water (Barton *et al.* 1952).

Other sprays used were: (a) 15% Na_2CO_3 followed by diazotized sulfanilic acid; (b) p-toluene sulfonic acid in ethanol; and (c) a mixture of 4 volumes of saturated ethanolic solution of vanillin to one volume of concentrated HCl.

Purification of the compounds

The spots containing the compounds were cut from the two-dimensional chromatograms, eluted with 90% methanol and concentrated under reduced pressure. Each concentrate was purified by paper chromatography four times using BAW, 2% acetic acid, water and 15% acetic acid, successively, as developing solvents. After each separation, the spot was eluted with 90% methanol and concentrated in vacuo.

Conversion of leucocyanidins to cyanidins

A portion of the purified compounds from spots L-1, L-2, L-3, L-4 and L-5 were each transferred to a test tube, 0.6 *N* HCl in n-butanol was added, and the tube was placed in a boiling water bath for 40 min.

The red pigments which appeared from this treatment were purified twice by descending paper chromatography using Forestal solvent (acetic acid–concentrated HCl-water, 30:3:10, v/v/v) the first time and n-butanol-2 *N* HCl (1:1, v/v) the second time.

The concentration of leucocyanidins in the ethyl acetate, aqueous and alcohol insoluble portions was determined by the method of Swain and Hillis (1959).

Acid hydrolysis of flavonols

A portion of the purified substances from spot K or Q was hydrolyzed in 2 *N* HCl for 20 min in a boiling water

bath. The aglycon was extracted with ethyl acetate and chromatographed twice using Forestal and BAW as developing solvents.

The aqueous fraction was concentrated and co-chromatographed descendingly with authentic glucose, galactose and rhamnose using two different solvent systems: BAW and ethyl acetate-acetic acid-water (3:1:3).

Absorption spectra

The UV and visible absorption spectra of the purified compounds and their acid-hydrolyzed products in methanol were obtained with a Bausch and Lomb Spectronic 505 recording spectrophotometer. The spectra were also obtained after addition of $AlCl_3$ and NaOAc + H_3BO_3 (Jurd, 1962).

RESULTS AND DISCUSSION

A drawing of the two-dimensional paper chromatogram of the ethyl acetate of the strawberry fruit is shown in Fig. 1. All spots were colored with the $FeCl_3 \cdot K_3Fe(CN)_6$ reagent, except spots 1, 2, 3 and 4. These four spots fluoresced under UV light and were useful in locating other phenolic compounds on the unsprayed paper.

Identification of some of the flavonoid compounds

1. *Leucocyanidins*

The UV spectra of the compounds eluted from spots L-1, L-2, L-3, L-4 and L-5 showed one peak at 280 mμ in absolute methanol. No shift of this peak was detected upon addition of $AlCl_3$.

When the eluates were heated in 0.6 *N* butanolic HCl in a boiling water bath, a red pigment was produced. This placed the compounds in the class of leucoanthocyanins. The red compounds were first compared paper chromatographically with cyanidin and pelargonidin using the Forestal irrigation solvent; all displayed the same R_f value as cyanidin, 0.47. They were then co-chromatographed with cyanidin employing two solvents: the Forestal solvent and the n-butanol-2 *N* NCl solvent; in both cases the unknown spots coincided with cyanidin; the R_f values were 0.47 and 0.73 for the respective solvents.

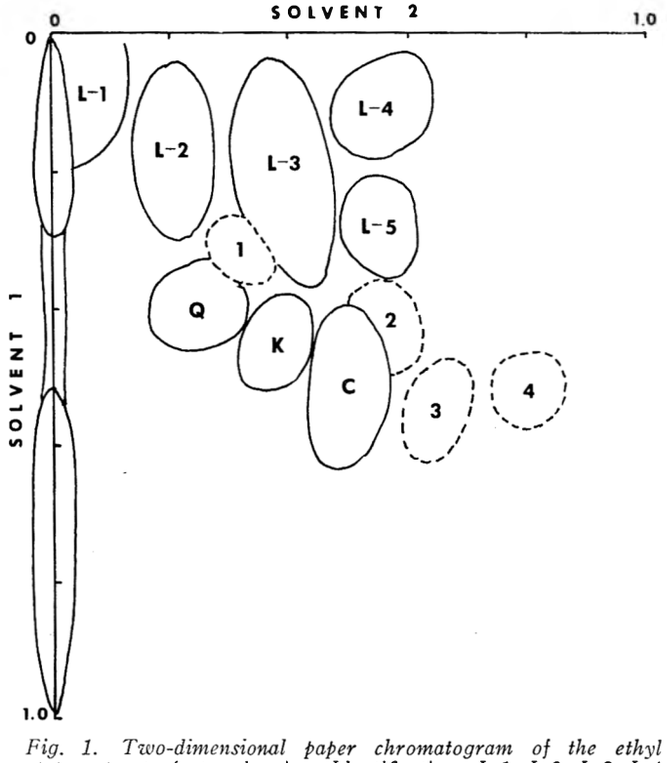

Fig. 1. Two-dimensional paper chromatogram of the ethyl acetate extract of strawberries. Identification: L-1, L-2, L-3, L-4 and L-5, leucocyanidins; Q, quercetin-3-glucoside; K, kaempferol-3-glucoside; C, catechin; 1, 2, 3 and 4, unidentified.

The visible absorption spectra of all red pigments in 95% ethanol showed a 545 mμ peak which was shifted to a longer wavelength upon addition of $AlCl_3$, an indication of the presence of an o-dihydroxyl group (Geissman *et al.*, 1953). The red pigments had the same spectral characteristics as cyanidin (Harborne, 1958). Therefore, spots L-1 to L-5 must be leucocyanidins. Their differential migration upon paper chromatography may indicate different degrees of polymerization. Govindarajan and Mathew (1963) reported a similar observation on the leucoanthocyanidins of Arecanut.

Conventionally (Clark-Lewis, 1965), leucoanthocyanidins are classified as: (a) those that are insoluble in water and lower alcohols, (b) those readily soluble in water and not extracted by ethyl acetate and (c) those extractible from aqueous solution by ethyl acetate. When this fractionation was applied to the leucocyanidins of the strawberry fruit and the concentration of the derived cyanidin was estimated photometrically, the results of Table 1 were obtained.

The differences between unripe and ripe strawberries regarding concentration of all types of these chromogens are rather small. It is interesting, however, that the water-soluble fraction, broadly representing glycosylated leucoanthocyanidins, is the largest of the three fractions.

2. *Flavonols*

Spots K and Q fluoresced yellow under 366 mμ UV light after exposure to NH_3 fumes, indicating that they might be flavonols. The fluorescent compounds were eluted, purified, acid-hydrolyzed, and the aglycons were paper-chromatographed. With the Forestal solvent the aglycon of compound Q had an $R_f = 0.42$ which was identical with that of quercetin; the aglycon of K displayed the R_f value of kaempferol, 0.58. Their R_f values in BAW were also the same with those of kaempferol, $R_f = 0.83$, and quercetin, $R_f = 0.64$. Co-chromatography with quercetin and kaempferol resulted in one spot for each case. The chromatographic data were corroborated by spectral findings. Known quercetin and kaempferol exhibited the same absorption peaks with the aglycons of spots Q and K. The wavelengths of these peaks along with the absorption

Table 1. Fractionation of the leucocyanidins of unripe and ripe strawberries. (Concentration as mg of cyanidin per 100 g fresh fruit.)

Fraction	Concentration	
	Unripe	Ripe
Water and methanol insoluble	22	20
Water soluble	29	32
Ethyl acetate extractible	16	21

maxima of the unhydrolyzed compounds are shown in Table 2.

Only glucose was found in the aqueous portion of the acid hydrolysates (Table 3).

The absorption peaks of the unhydrolyzed spots K and Q were in agreement with reported data (Jurd, 1962). When $AlCl_3$ was added, a bathochromic shift was observed in both compounds. This shows that the 5-OH or 3-OH is free. When NaOAc was added, a bathochromic shift was also observed which shows that the 7-OH is free. When NaOAc + H_3BO_3 was added, the long wavelength peak of Q showed a shift from 360 to 377 mμ indicating the presence of o-dihydroxyl group. The fact that spots K and Q remained dark under UV light (366 mμ) shows that the 3-OH is not free.

The R_f values of the glucosides of quercetin and kaempferol in BAW were 0.44 and 0.49, respectively, and in 2% acetic acid 0.21, 0.30, respectively. These values were identical with those of quercetin-3-glucoside and kaempferol-3-glucoside and were verified by co-chromatography.

Table 2. Absorption peaks of strawberry flavonols in methanol, before and after addition of $AlCl_3$, NaOAc and NaOAc + H_3BO_3.

	max (mμ)			
	Aglycon of compounds		Compounds	
Solvent	Q	K	Q	K
Methanol	257,371	267,367	258,360	268,352
Methanol + $AlCl_3$	272,426	270,431	267,418	270,428
Methanol + NaOAc	275,395	275,388	272,395	275,380
Methanol + NaOAc + H_3BO_3	260,389	267,368	261,377	267,352

Table 3. R_f values of the sugar moieties of strawberry flavonols.

Samples	Ethyl acetate–acetic acid–water (3:1:3)	n-Butanol–acetic acid–water (6:1:2)
Acid hydrolysate of spot K	0.15	0.22
Acid hydrolysate of spot Q	0.15	0.22
Glucose	0.15	0.23
Galactose	0.13	0.21
Rhamnose	0.32	0.44

3. *Catechin*

Spot C appeared dark under UV light of short wavelength (254 mμ) and showed blue fluorescence under UV light of long wavelength (366 mμ), after exposure to NH_3 fumes. When co-chromatographed with known catechin one spot appeared with the R_f of 0.39 in distilled water. When the paper chromatogram was sprayed with the $FeCl_3$-$K_3Fe(CN)_6$ reagent, spot C became blue. When a separate chromatogram was sprayed with the vanillin-HCl reagent, the spot turned red. Both of these color reactions are given by catechin.

The identity of the spot C was further confirmed by its absorption spectrum in the UV region. The maximum absorption peak in ethanol appeared at 280 mμ.

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R. K. CREVELING,[a] R. M. SILVERSTEIN [b] and W. G. JENNINGS[a]

Volatile Components of Pineapple

SUMMARY—By gas chromatographic retentions, infrared spectroscopy and where applicable, mass and nmr spectroscopy, several additional compounds have been identified as components of pineaapple essence. These are acetoxyacetone, dimethyl malonate, *trans*-tetrahydro-α,α,5-trimethyl-5-vinyl furfuryl alcohol, methyl *cis*-(4?)-octenoate, γ-butyrolactone, methyl β-hydroxybutyrate, methyl and ethyl β-hydroxyhexanoate, methyl and ethyl β-acetoxyhexanoate, γ-octalactone, and δ-octalactone. Possible biogenetic pathways to some of these compounds are discussed.

INTRODUCTION

BECAUSE IT POSSESSES both a strong, characteristic aroma and considerable economic significance, pineapple has been studied extensively. This has resulted in the isolation and chemical characterization of a variety of compounds from pineapple essence (Haagen-Smit *et al.*, 1945; Gawler, 1962; Connell, 1964; Rodin *et al.*, 1965; Silverstein *et al.*, 1965; Rodin *et al.*, 1966). The present work extends our knowledge of pineapple composition, and draws on this and previous knowledge to suggest possible biogenetic pathways for the production of some of these materials.

METHODS AND PROCEDURE

ETHER EXTRACTS OF PINEAPPLE, *Ananas comosus* (L.) Merr. var. Smooth Cayenne, were prepared from freshly harvested fruits by the Pineapple Research Institute of Hawaii (Rodin *et al.*, 1965). Since these extracts contained considerable amounts of non-volatile material, they were distilled in four steps at pressures from atmospheric to 20μHg, during which the pot temperature was never allowed to exceed 50°C. A Podbielniak column was used for the atmospheric distillation. A water aspirator and a single stage oil vacuum pump were used for subsequent distillations.

The distillate was condensed with traps cooled with wet ice and dry ice-alcohol; all connections were Teflon and glass. Finally, a cold-finger molecular still was used to recover some of the less volatile components from the pot residue. In all cases, dry ice-alcohol traps were used subsequent to the collection traps to prevent contamination from the pumping apparatus. The distillates were combined, dried over anhydrous $MgSO_4$, and most of the ether was removed with the Podbielniak column.

Preliminary gas chromatographic fractionation of the distillate utilized an Aerograph Autoprep containing an 18-ft × ¼-in. stainless steel column packed with 10% Triton X-305 on 60 to 80 mesh HMDS treated Gas Chrom Q. The unit had a flow rate of 47 cc/min and was programmed from 60 to 205°C; only those fractions whose retentions exceeded that of acetic acid were collected for this study. These 16 fractions were rechromatographed at a flow rate of 6 cc/min on 500-ft × 0.03-in. open tubular columns coated with SF96(50) (admixed with 5% Igepal CO-880), or Carbowax 20M. These latter separations were performed at various isothermal conditions. Fractions were collected in 12-in., thin-wall glass capillary tubing, inserted in the exit port of the chromatograph. A seal and a head gradient were provided by tightly wrapping a small triangle of aluminum foil around the inserted end of the tube.

Infrared spectra were obtained on a Beckman IR-8 infrared spectrophotometer by eluting the condensed material from the collecting tube with 0.6–0.8 μl methylene chloride, and slowly depositing the solution onto a sodium chloride sandwich type cell. The methylene chloride rapidly evaporated, leaving a thin film. When enough material was available, and gas chromatographic retentions and infrared spectroscopy yielded inconclusive results, samples were subjected to mass (CEC21-103C) and nuclear magnetic resonance spectrometry (Varian HA-100). The spectral and retention data on the isolated compounds are given in Figs. 1–12.

The authentic compounds used for chromatographic and infrared comparisons were obtained as follows: acetoxyacetone was purchased from Aldrich Chemical Co., Milwaukee, Wisc.; dimethyl malonate and γ-butyrolactone were purchased from K & K Laboratories, Plainview, N. Y.; *trans*-tetrahydro-α,α,5-trimethyl-5-vinyl furfuryl alcohol was prepared by Dr. C. S. Tang, University of Cali-

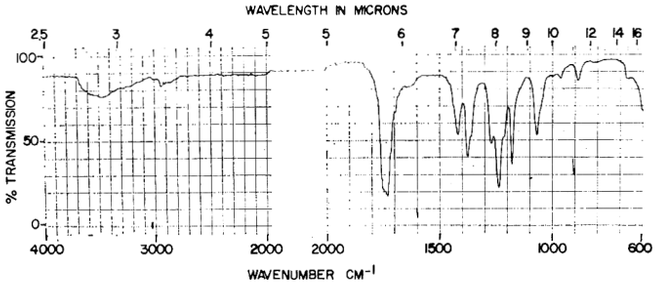
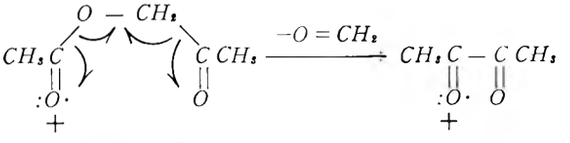


Fig. 1. Compound 21A. Acetoxyacetone.
GLC retention: *Triton X-305, 131°, 39 min; SF-96(50), 106°, 19 min.*
Mass spectrum *(diagnostic peaks): 116(P), 74 (P−CH_2=C=O), 73 (P−CH_3C=O), 43 (CH_3C=O). A prominent peak at m/e 86 may be accounted for as follows:*



NMR spectrum *(γ, CCl_4): 7.95 (2 peaks almost coincident, 6 protons, CH_3C=O and CH_3COO), 5.58 (singlet, 2 protons, $COOCH_2$C=O).*

[a] Department of Food Science and Technology, University of California, Davis.
[b] Stanford Research Institute, Menlo Park, California.

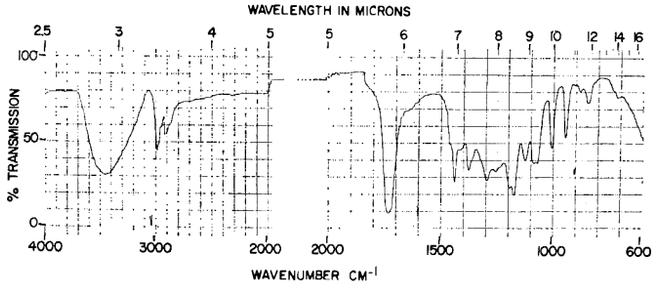


Fig. 2. Compound 22A. Methyl β-hydroxybutyrate.
GLC retention: *Triton X-305, 134°, 41 min; SF-96 (50), 105°, 19.3 min.*
Mass spectrum *(diagnostic peaks): 118 (P, very weak), 117 (P—H), 103 (P—CH_3), 100 (P—H_2O). 87 (P—OCH_3), 85 (P—CH_3 and H_2O), 74 (CH_2COOCH_3 + H), 69 (—H_2O and OCH_3), 59 (P—$COOCH_3$).*

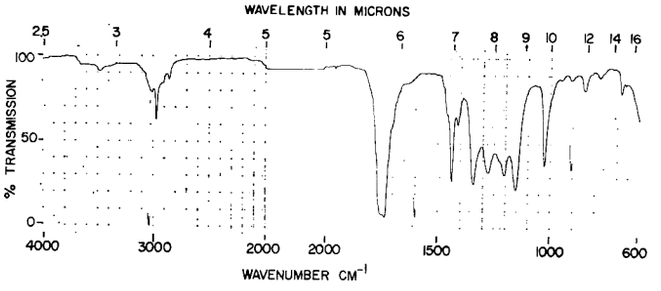


Fig. 3. Compound 23A. Dimethylmalonate.
GLC retention: *Triton X-305, 137°, 42 min; SF-96(50), 121°, 18.3 min.*

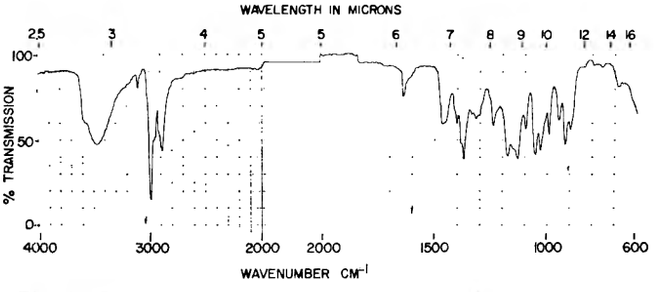


Fig. 4. Compound 23D. trans *Tetrahydro-α,α,5-trimethyl-5-vinyl furfuryl alcohol.*
GLC retention: *Triton X-305, 137°, 42 min; SF-96(50), 121°, 26.2 min.*

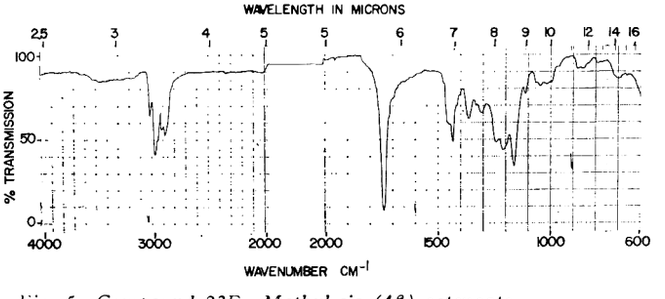


Fig. 5. Compound 23F. Methyl cis-*(4?)-octenoate.*
GLC retention: *Triton X-305, 137°, 42 min; SF-96(50), 121°, 28.8 min.*
Mass spectrum *(diagnostic peaks): 156 (P), 125 (P—OCH_3), 124 (P—CH_3OH), 97 (P—$COOCH_3$).*

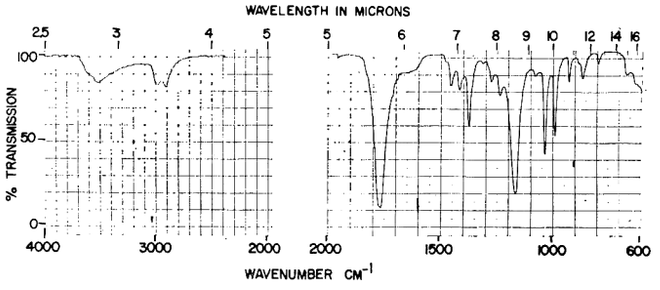


Fig. 6. Compound 28A. γ-Butyrolactone.
GLC retention: *Triton X-305, 150°, 50 min; SF-96(50), 132°, 17.5 min.*

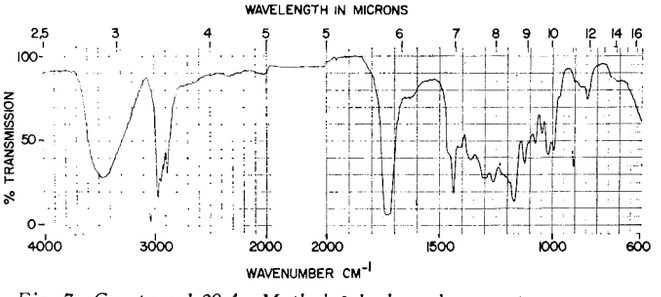


Fig. 7. Compound 29A. Methyl β-hydroxyhexanoate.
GLC retention: *Triton X-305, 152°, 51 min; SF-96(50), 126°, 22.3 min.*
Mass spectrum *(diagnostic peaks): 146 (P, very weak), 145 (P—H), 128 (P—H_2O), 115 (P—OCH_3), 113 (P—H_2O and CH_3), 103 (P—$CH_3CH_2CH_2$), 97 (P—H_2O and OCH_3).*
NMR spectrum *(γ, CCl_4): 9.07 (distorted triplet, 3 protons, CH_3CH_2), 8.58 (multiplet, 4 protons $CH_3CH_2CH_2$), ~7.7 (overlapping pattern, 2 non-identical protons, C*—CH_2—C=O, near center of asymmetry), 7.35 (slightly broadened singlet, 1 proton, OH) 6.38 (singlet, 3 protons, OCH_3), 6.15 (multiplet, 1 proton, CHOH).*

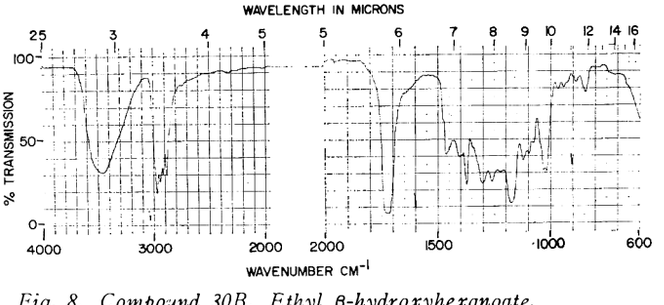


Fig. 8. Compound 30B. Ethyl β-hydroxyhexanoate.
GLC retention: *Triton X-305, 155°, 54 min; SF-96(50), 127°, 27.0* min.
Mass spectrum *(diagnostic peaks): 160 (P. very weak), 159 (P—H), 142 (P—H_2O) 131 (P—CH_2CH_3), 117 (P—$CH_2CH_2CH_3$), 115 (P—OCH_2CH_3), 97 (P—H_2O and CH_3CH_2O), 88 ($CH_2COOCH_2CH_3$ + H), 73 ($COOCH_2CH_3$ and $CH_3CH_2CH_2CHOH$), 72, (73—H), 71 (73—H_2), 55 ($CH_3CH_2CH_2CHOH$—H_2O).*

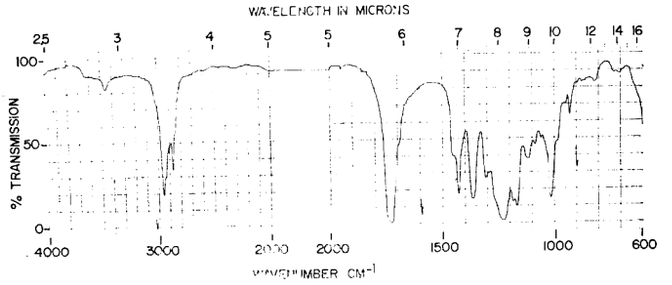


Fig. 9. Compound 31E. Methyl β-acetoxyhexanoate.
GLC retention: *Triton X-305, 156°, 55 min; SF-96(50), 133°, 25.7 min.*
Mass spectrum *(diagnostic peaks): 188 (P, not observed), 157 (P—OCH_3, weak), 145 (P—$CH_3C{=}O$, and P—$CH_3CH_2CH_2$), 128 (P—CH_3COOH), 97 (P—CH_3COOH and OCH_3), 74 (CH_2COOCH_3 + H).*
NMR spectrum *(γ, CCl_4): 9.10 (distorted triplet, 3 protons, CH_3CH_2)/ ~8.30 — 8.85 (multiplet, 4 protons, CH_2CH_3), 8.19 (singlet, 3 protons, CH_3COO), 7.58 (doublet, 2 protons, CH_2-COO), 6.42 (singlet, 3 protons, $COOCH_3$), 4.95 (quintet, 1 proton, CH_3COOCH).*

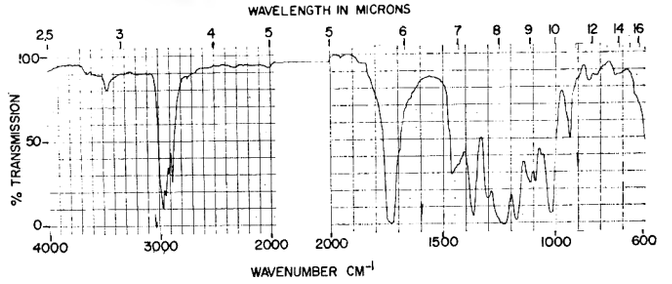


Fig. 10. Compound 33B. Ethyl β-acetoxyhexanoate.
GLC retention: *Triton X-305, 160°, 58 min; SF-96(50), 138°, 31.0 min.*
Mass spectrum *(diagnostic peaks): 202 (P, very weak), 160 (P — $CH_2{=}C{=}O$), 159 (P — $CH_3C{=}O$ and P — $CH_3CH_2CH_2$), 157 (P — OCH_2CH_3), 142 (P — CH_3COOH), 97 (P — OCH_2-CH_3 and CH_3COOH), 88 ($CH_2COOCH_2CH_3$), 73 ($COOCH_2$-CH_3), 43($CH_3C{=}O$ and $CH_3CH_2CH_2$).*

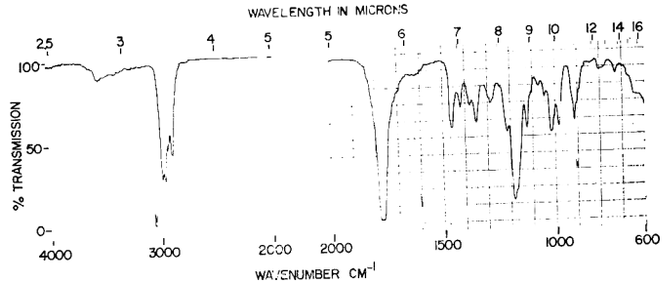


Fig 11. Compound 37B. γ-Octalactone.
GLC retention: *Triton X-305, 190°, 71 min; SF-96(50), 160°, 22.0 min.*

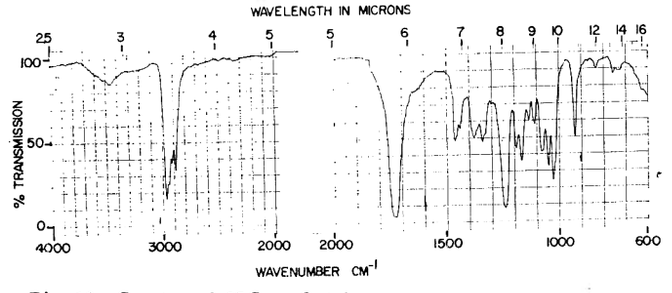


Fig. 12. Compound 37C. δ-Octalactone.
GLC retention: *Triton X-305, 190°, 71 min; SF-96(50), 160°, 23.3 min.*

fornia, Davis; γ- and δ-octalactones were supplied by Dr. R. E. Wrolstad, Oregon State University; the methyl β-hydroxy- and acetoxyhexanoates were synthesized by published methods (Shriner, 1942).

RESULTS AND DISCUSSION

FIGURES 1–12 REPORT the compounds isolated and identified in this investigation. There were at least five other fractions having infrared spectra very similar to those of the β-hydroxy- and acetoxyhexanoate esters and methyl β-hydroxybutyrate, suggesting that a series of related compounds may be present. Heinz *et al.* (1966) identified methyl β-hydroxyoctanoate as a constituent of Bartlett pear, and postulated it might arise through a β-oxidation cycle during fruit ripening. This possibility was supported by an abundance of unsaturated fatty acid esters that could also be fitted into the cycle. Compounds of the latter type do not appear to be abundant in pineapple, but β-hydroxy acid moieties could also arise from fatty acid synthesis. Although the odors of the isolated β-hydroxy esters are rather repulsive, their intensities suggest that these esters are important to pineapple aroma. The methyl and ethyl β-hydroxy hexanoates have also been found in Valencia orange juice by Schultz *et al.*, 1964.

Tetrahydro-α,α,5-trimethyl-5-vinyl furfuryl alcohol, having the trans configuration with respect to the ring, has been isolated from grapes (Stevens *et al.*, 1966) and apricots (Tang and Jennings, 1967). In each of these cases, linalool was also found and postulated to be the precursor. So far, linalool has not been isolated from pineapple. The octalactones exhibit the coconut-like aroma typical of many other lactones. Relatively large amounts of γ- and δ-lactones have been found in peaches (Jennings *et al.*, 1964) and apricots (Tang *et al.*, 1967). The *cis*-(4?)-octenoate moiety is probably associated with the octalactones.

Mention has been made that many compounds isolated from fruits have the same moieties as the intermediates in established catabolic and anobolic fatty acid pathways of other organisms (Conn *et al.*, 1966). Although the ripening of fruit has been the subject of much research, little attention, aside from ethylene, has been paid to the role volatile constituents play in the intermediate metabolism at this period of the fruit's life.

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JOHN A. ATTAWAY and M. F. OBERBACHER
Florida Citrus Commission and University of Florida Citrus Experiment Station
P.O. Box 1088, Lake Alfred, Florida 33850

Studies on the Aroma of Intact Hamlin Oranges

SUMMARY—Two approaches were used in a study of compounds contributing to the aroma of Hamlin oranges, which had been carefully handled to prevent release of peel oil. Volatile aromatic compounds emitted from the oranges on storage, and less volatile aroma compounds present on the cuticle of the fruit were isolated and analyzed. The volatile aroma of the stored oranges seemed to be contributed by ethyl esters, particularly ethyl butyrate, while sesquiterpene hydrocarbons appeared to be responsible for the persistent odor from the cuticle of fresh oranges. Volatile compounds definitely identified include ethyl acetate, ethanol, ethyl butyrate, limonene, ethyl caproate and ethyl caprylate. The sesquiterpene hydrocarbons on the cuticle consisted chiefly of valencene, with lesser amounts of elemene, caryophyllene, farnesene, humulene and cadinene.

INTRODUCTION

ALTHOUGH THE LITERATURE provides reports of research on the identification of the constituents of orange peel oil and the volatile aroma compounds associated with orange juice (Kirchner *et al.*, 1957; Wolford *et al.*, 1963; Attaway *et al.*, 1964) very little has been done on the identification of compounds responsible for the aroma of intact oranges where released peel oil is not a factor. Probably the reason for this omission lies in the fact that intact oranges possess only a slight aroma when contrasted with such highly aromatic fruits as apples and pears.

This research reports the results of two approaches toward the identification of orange aroma based on different observations. First, it has been noted that when boxes of stored Hamlin oranges were opened for examination, a pleasant, fruity aroma was present. This was particularly true when samples of this early-maturing variety were picked when fully mature. From this it was concluded that volatile, aromatic compounds were emitted from the oranges during storage and trapped within the boxes so that a detectable concentration accumulated. Accordingly, the first approach involved trapping these emitted volatiles by passing dry air over stored fruit for a period of several days so that the compounds producing the fruity aroma could be swept out and collected on activated charcoal.

The second observation was that clean, fresh oranges possess a characteristic odor quite different from the fruity aroma produced by the compounds emitted on long storage. It was further noted that this is a very persistent odor that can be detected on the hands for up to an hour or more after handling the oranges. This indicated the presence of relatively high-boiling compounds of low volatility. Consequently, the second approach involved washing of oranges in solvent to remove and concentrate these compounds.

EXPERIMENTAL

Study of emitted volatiles

Preparation of fruit. The fruit was hand-picked using clippers and handled gently to prevent skin rupture and subsequent release of peel oil. The stem ends were painted with a 5% aqueous solution of thiourea to retard decay.

Collection of emitted compounds. Volatile aroma compounds emitted by the oranges were collected on activated charcoal. In a typical run, duplicate samples of 70 fruit (approximately 9 kg) were placed in 5-gallon glass jars fitted with inlet and outlet tubes and stored at 70°F. Air from a compressed air cylinder was brought through a drying tower containing activated charcoal, a drying tube containing drierite (anhydrous $CaSO_4$), and then circulated through each jar at a rate of 15–20 ml/min. The jars were connected in parallel, and the outlet of each was fitted with 2 U-tubes in series packed with 20 g activated coconut charcoal (Fisher Scientific Co., 6–14 mesh) per tube. The air flow was maintained for 12 days, after which the

charcoal from all 4 traps was combined and extracted for 3 days with diethyl ether (Baker Analyzed Reagent) in a Soxlet extractor. The ether was removed by slow evaporation using a Rinco rotary evaporator (Rinco Instruments, Inc., Greenville, Ill.) and aspirator vacuum. About ½ ml of ether-rich material remained, which had a fruity odor strongly reminiscent of ethyl butyrate.

Gas chromatographic analysis. Gas liquid chromatographic analysis (GLC) was carried out on two different column phases of opposite polarity using an F & M Model 810 dual-column, temperature-programmed instrument with flame ionization detection. The polar column, designated column A, was 50 ft × ⅛-in. and contained 5% Carbowax 20 M on 60–80 mesh Gas Chrom Z. The non-polar column, designated column B, consisted of 5% Apiezon L plus Igepal CO-710 (20 parts Apiezon—1 part Igepal) on 60–80 mesh Gas Chrom Z.

The chromatogram in Fig. 1 was prepared using column A. Aroma volatiles in ether (0.5 microliter) were injected into the instrument with the column temperature at 70°C. The temperature was held at 70°C for 10 min and then programmed to 180°C at a rate of 4°C/min. The attenuation was $10^2 \times 4$ to bring out small peaks, but was increased to as high as $10^2 \times 64$ to keep the major aroma volatile peaks on scale.

The chromatogram in Fig. 2 was prepared using column B. Again, 0.5 μl of aroma volatiles were injected onto the column at 70°C and the program rate was 4°C/min following an initial hold period of 10 min. However, in this case the attenuation was held at $10^2 \times 32$ for the entire chromatogram.

Mass spectral analysis. Mass spectra were prepared using a Perkin-Elmer Hitachi mass spectrometer coupled directly to the gas chromatograph.

Study of aroma compounds present on cuticle of fresh oranges

Preparation of fruit. The fruit for this study were also carefully hand-clipped. Each of 160 fruit used was washed gently with a laboratory detergent, dried with cheesecloth, and allowed to stand for 24 hr to replenish aroma compounds lost during washing.

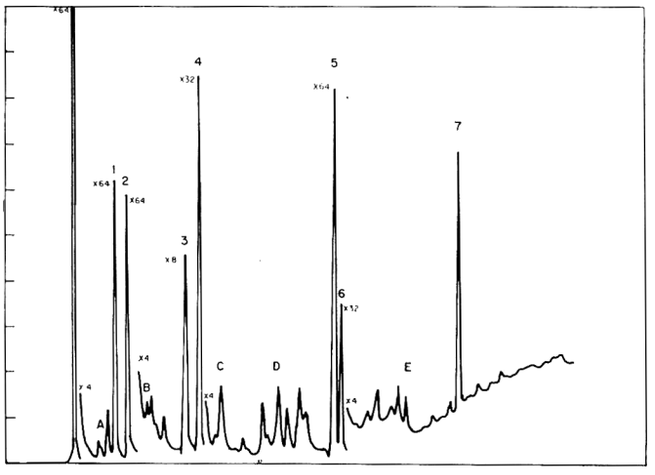

Fig. 1. Gas chromatogram of emitted volatiles obtained using polar column. Compounds corresponding to numbers are as follows: 1. ethyl acetate, 2. ethanol, 3. unknown of m.w. 114, 4. ethyl butyrate, 5.(+)-limonene, 6. ethyl caproate, and 7. ethyl caprylate. Compounds corresponding to letters are as follows: A. ethyl formate, B. ethyl propionate, C. ethyl isovalerate, D. ethyl valerate, and E. ethyl heptoate.

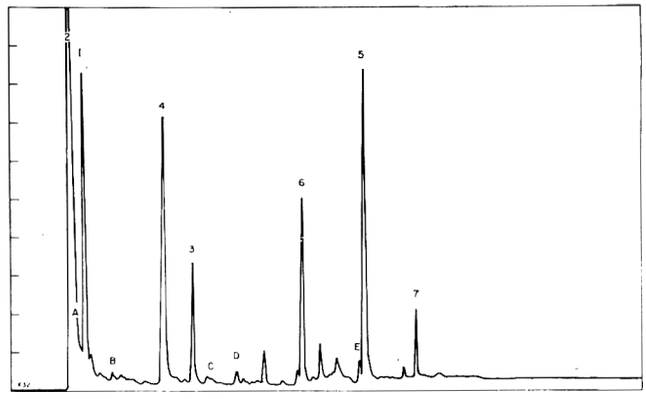

Fig. 2. Gas chromatogram of emitted volatiles obtained using non-polar column. Compounds corresponding to the numbers and letters are the same as in Fig. 1.

Extraction and concentration of volatiles. Two liters of methylene chloride (Matheson, Coleman, and Bell) was placed in a large beaker, and each fruit was dipped in the solvent using a pair of laboratory tongs. Great care was taken in handling the fruit to insure that the peel was not scraped or cut with the resultant release of peel oil. The methylene chloride was then filtered through Whatman #1 paper to remove scale insects and other solid particles that had escaped the detergent wash, following which it was concentrated on a rotary evaporator. About a milliliter of methylene chloride-rich solution remained, which possessed a strong odor of fresh oranges.

Gas chromatographic analysis. GLC was carried out on an F & M Model 500 dual-column, temperature-programmed gas chromatograph with thermal conductivity detection. The column was 12 ft × ¼ in of 20% Carbowax 20M on 60–80 mesh GasChrom P. Ten microliters of solution was injected on the column at 70°C following which it was programmed to 200° at 4°/min. It was held at 200° until all the components had emerged. The chromatogram is shown in Fig. 3.

To clean and concentrate the sesquiterpene material, 15–40 μl collecting runs were made on the same column. The resulting sesquiterpene fraction was rechromatographed on the F & M Model 810 gas chromatograph

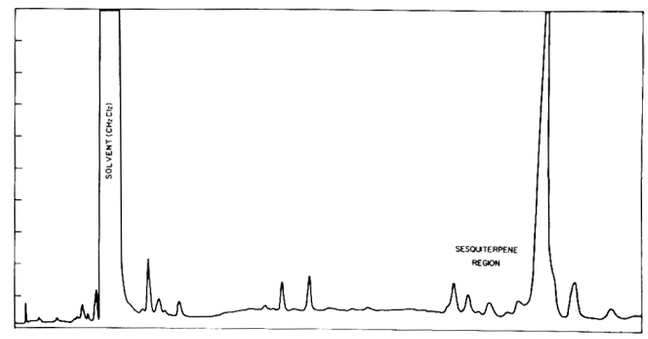

Fig. 3. Gas chromatogram of methylene chloride solution obtained by dipping fresh oranges.

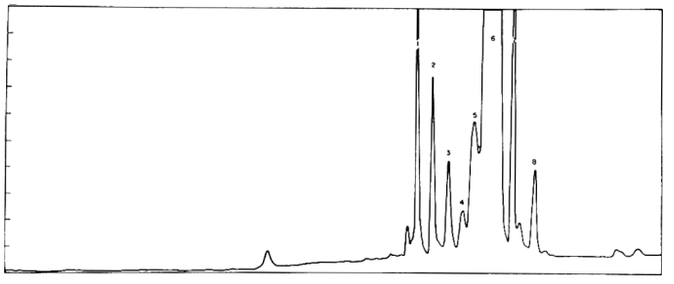

Fig. 4. Gas chromatogram of sesquiterpene cut from cuticle of fresh oranges. Compounds corresponding to the numbers are as follows: 1. beta-elemene, 2. beta-caryophyllene, 3. farnesene, 4. humulene, 5. $C_{15}H_{24}$, 6. valencene, and 7. delta-cadinene.

using column A as described previously. The chromatogram is shown in Fig. 4.

RESULTS AND DISCUSSION

Study of emitted volatiles

The ether extract from the activated charcoal was found to contain 7 major components, which were identified by gas liquid chromatography on 2 column phases and by mass spectrometry. These are numbered 1–7 in order of their emergence from GLC column A as shown in Fig. I. The major peak going off scale near the beginning of this chromatogram is the solvent, diethyl ether. The major numbered compounds are 1) ethyl acetate, 2) ethanol, 3) an unidentified compound of molecular weight 114, 4) ethyl butyrate, 5) (+)−limonene, 6) ethyl caproate, and 7) ethyl caprylate. The relative quantities of each may be estimated from the attenuation values shown by the small numbers preceeded by x. The presence of ethanol may be due to microorganism activity over the long period of the experiment, while limonene must originate in the peel oil. The ethyl esters are probably responsible for the fruity aroma, which was originally described as reminiscent of ethyl butyrate. The molecular weight 114 material appeared from it's mass spectrum to be a ketone; however, it could also be an ester.

In addition, several minor peaks of the chromatograms coincided with other ethyl esters. These are shown on the chromatograms by letters, but their identification is uncertain because they were not present in a sufficiently large concentration for mass spectrometric verification. The compounds in question are A) ethyl formate, B) ethyl propionate, C) ethyl isovalerate, D) ethyl valerate, and E) ethyl heptoate.

When the chromatographic analyses were carried out at higher temperatures and over longer time periods, it was found that ionol, commonly known as butylated hydroxy toluene (BHT), eluted from the column. It was positively identified by its mass spectrum. To determine if this compound was an artifact, a sample of the ether used in the extraction was evaporated to dryness, but no BHT was found. A large quantity of the coconut charcoal was then extracted and the extract analyzed, but, again, no BHT was found, indicating the possibility that BHT may be a natural constituent of oranges. However, this should be investigated further.

Study of aroma compounds present on cuticle of fresh oranges

The methylene chloride extract was found to consist almost entirely of sesquiterpene hydrocarbons, although the monoterpene, sabinene, was identified as a trace constituent of the forerun. The predominant sesquiterpene was valencene, peak 6 of Fig. 4, which was estimated to contribute over 90% of this fraction. Five other sesquiterpenes were tentatively identified as beta-elemene, beta-caryophyllene, farnesene, humulene, and delta-cardinene, peak numbers 1, 2, 3, 4, and 7 respectively. These compounds appear to be major contributors to the characteristic odor of fresh oranges.

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K. E. MURRAY, J. SHIPTON, F. B. WHITFIELD, B. H. KENNETT and G. STANLEY
Division of Food Preservation
Commonwealth Scientific and Industrial Research Organization
Ryde, N.S.W. 2112, Australia

Volatile Flavor Components from Green Peas (Pisum Sativum). 1. Alcohols in Unblanched Frozen Peas

SUMMARY—The volatile alcohols of unblanched frozen green peas were separated from the total volatiles by chromatography on silica gel at 0°C and examined by combined gas chromatography-mass spectrometry and where possible by infra-red spectroscopy. Thirteen saturated and nine unsaturated alcohols were positively identified: methanol, ethanol, propan-1-ol, butan-1-ol, butan-2-ol, 2-methylpropan-1-ol, pentan-1-ol, pentan-3-ol, 2-methylbutan-1-ol, 3-methylbutan-1-ol, hexan-1-ol, heptan-1-ol, octan-1-ol, pent-1-en-3-ol, *cis* and *trans* pent-2-en-1-ol, *cis* and *trans* hex-3-en-1-ol, *trans* hept-2-en-1-ol, *trans* oct-2-en-1-ol, oct-1-en-3-ol and *cis* non-3-en-1-ol. Several other alcohols were also present and, of these, *trans* hex-2-en-1-ol, *cis* and *trans* hept-3-en-1-ol and nonan-1-ol were tentatively identified.

The interconnecting system between gas chromatograph and mass spectrometer permitted the collection, for IR analysis, of material from the identical gas chromatographic peak on which the mass spectrum was obtained.

INTRODUCTION

COMMERCIALLY PRODUCED frozen green peas often possess a "haylike" off-flavor. This is associated with a characteristic odor which strongly suggests that the off-flavor is due to volatile compounds. However, as yet, no evidence has been produced to indicate the organoleptically significant compounds, although a number of constituents of pea volatiles has been identified (Ralls, 1960; Bengtsson *et al.*, 1964; Ralls *et al.*, 1965; Whitfield *et al.*, 1966). Investigations in this laboratory are being directed to determine the nature of the normal and "off" pea flavor.

It is well recognized that, despite the sophisticated instrumental techniques now available, a full understanding of any volatile food flavor is not easily achieved. The experimental procedures to accomplish this may be conveniently divided into three inter-dependent stages:

(a) the isolation and concentration of the volatile complex free from artifacts and contaminants,
(b) the separation and identification of the volatile constituents, and
(c) the assessment of the organoleptic significance of the constituents, both individually and collectively.

As both Stewart (1963) and Burr (1964) have emphasized, the identification of food volatile constituents is of limited value unless followed by a conclusive organoleptic evaluation. This, however, cannot be effectively undertaken unless the identification of constituents has been thorough, and this is contingent on having sufficient amounts of volatile flavor concentrates for the identification of the minor constituents.

The low level of volatiles present in peas has primarily dictated the choice of techniques for identification of constituents. It was apparent at the outset of this work that if the amount of raw material to be extracted was to be kept within practical limits, then, for the majority of compounds, identification would depend largely on mass spectral evidence.

Combined gas chromatography-mass spectrometry (GC-MS) clearly offers the best means of recording the mass spectra of minimal size samples. However, often the quality of spectra obtained is impaired by:

(a) incomplete resolution of components, thus giving mixed spectra,
(b) distortion of the spectrum due to the inevitable rapid change in concentration which occurs during the scan, and/or
(c) interference from the background spectrum caused by phase bleed from the column.

The measures taken in the current work to minimize or eliminate these deficiencies are described.

The volatile content of peas increases rapidly during the interval between mechanical harvesting (vining) and enzyme inactivation by blanching in boiling water (Shipton *et al.*, 1968). However, blanching, while markedly reducing the concentration of volatiles in the peas, does not change their qualitative composition as judged by gas chromatograms (Whitfield *et al.*, 1967). Therefore, unblanched peas, which had been held for some hours at ambient temperature before freezing, were used in the development of extraction techniques and for the initial studies on pea flavor. The identification of the volatile constituents of unblanched peas will facilitate the investigation of fresh and blanched peas, in which the volatiles occur at much lower concentrations.

EXPERIMENTAL PROCEDURES

GREEN PEAS (*Pisum sativum*, variety "Edgell Freezer") grown at Richmond, N.S.W., were harvested, vined, washed and frozen. The delay period between harvesting and freezing varied from 4 to 6 hr with different batches. The frozen peas were stored (6–8 months) at −17.8°C.

Isolation of volatile flavor concentrate

The frozen peas, in batches of 6 kg, were rapidly minced into a 10-l round bottom flask and the volatile material distilled through cold (0°C) reflux condensers for 5 hr at 8 mm pressure, the distillate being collected in a liquid air-cooled trap (Whitfield *et al.*, 1967). The flask was warmed by a water bath at 20°C and in the course of the distillation the temperature of the continuously stirred

puree rose from −18 to 15°C. The distillate, after thawing, was saturated with analytical grade sodium chloride and stored at 0°C. Sixty kg of peas yielded 600 ml of distillate.

The distillate was then fractionated by a previously described procedure (Shipton *et al.*, 1966) to separate the large excess of ethanol from the less volatile components. Gas chromatographic examination indicated that the ethanolic fraction also contained small amounts of methanol and other lower boiling compounds. The less volatile fraction was isolated, essentially water-free, as an oil (0.5 ml) which contained a relatively small proportion of ethanol. It had an aroma similar to, but much more intense than, that of the freshly minced frozen peas.

Class separation of the alcohols

A preliminary examination of the oil by GC-MS showed the presence of small amounts of C_{10} – C_{15} paraffinic hydrocarbons which detracted from the clarity of the spectra of those alcohols from which they were incompletely resolved. Accordingly, the alcohols as a class were separated from the less polar constituents by liquid chromatography on silica gel at 0°C. The technique used (Murray *et al.*, 1968) was designed to handle small amounts (5–50 mg) of volatile flavor concentrates.

The separated alcohol fraction showed the presence of many unsaturated alcohols. These were generally at a lower concentration than the saturated alcohols and in some cases incompletely resolved from them. To obtain better resolution of the unsaturated alcohols, the alcohol fraction was further fractionated by liquid chromatography on silver nitrate-silica gel (Murray *et al.*, 1967). The resulting fractions, when subjected to GC-MS, gave satisfactory resolution of all the alcoholic constituents with the exception of 2- and 3-methylbutan-1-ols.

Gas chromatography

A flame ionization detector was used. Details of the columns (stainless steel), their operating conditions and their respective uses follow:

Column A.
67 ft, 0.107 in I.D.
3.75% FFAP (Varian Aerograph) on 60–70 mesh non-acid washed Chromosorb G.
Isothermal 130°C.
10 ml/min helium.
Used for preliminary examination of all alcohol fractions and for coupling to the mass spectrometer to obtain spectra of components with retention times up to that of octan-1-ol.

Column B.
5 ft, 0.092 in I.D.
4.75% FFAP on 100–120 mesh non-acid washed Chromosorb G.
Isothermal 110°C.
8 ml/min helium.
Coupled to the mass spectrometer to examine components with retention times exceeding that of heptan-1-ol.

Column C.
18 ft, 0.065 in I.D.
5% Carbowax 200 on Aeropak 30.
Isothermal 60°C.
10 ml/min helium.
Used to resolve 2- and 3-methylbutan-1-ols (Singer, 1966) from the mixed peak collected from effluent of Column A. The phase bleed was too great to permit connection to the mass spectrometer.

Mass spectrometry

The mass spectrometer, an ATLAS CH4 (Fried Krupp, Germany) was fitted with a double ion source (Brunnée *et al.*, 1963). One chamber of this source, operated at 70 eV, projects the ion beam into the analyzer tube to produce the mass spectrum, while the other chamber, operated at 20 eV to avoid ionizing helium, monitors the total ion current. The double ion-source was modified in in the following respects:

(a) the flexible connection between the source and the total ion current preamplifier was replaced by a rigid connection to reduce the noise level, and

(b) a suppressor electrode operated at −85 V was installed adjacent to the total ion current collector electrode to minimize the undesirable effect of secondary electrons emitted from the collector electrode. This electron emission, influenced by the changing magnetic field, had caused variations in the total ion current during scanning.

Considerable tailing of polar compounds, particularly alcohols, occurred in the original ATLAS GC-MS system. This was attributed to adsorption on the stainless steel connecting valves and tubing. It was especially evident after "baking out". The problem was overcome by providing a direct connection between the gas chromatographic column and the ion source. (It is understood that a comparable modification has been incorporated by Fried Krupp in recent CH4 instruments.) Part (Tube B, Fig. 1) of this connection forms the capillary leak of the mass spectrometer and was constructed from 15 in. of stainless steel tube (0.0225 in. bore) into which was inserted the same length of stainless steel wire (0.0220 in.). One end of the leak tube passes through the ion source hood and projects into the ion source inlet block while the other end is located in the gas chromatographic column effluent. The leak tube is supported within a 0.25 in O.D. stainless steel tube, which is electrically heated. When operated at 140–180°C, approximately 0.5 ml/min of column effluent passed to the ion source. No adsorption by this capillary connection has been observed and no blockage, due to phase bleed, has occurred.

The column effluent is first divided by a splitter located in the gas chromatographic oven, about 30% passing to a flame ionization detector. As indicated in Fig. 1, the remainder passes over the end of the mass spectrometer leak tube B at point A where 0.5 ml/min is withdrawn. The residual effluent then flows through the narrow annulus between the leak tube and tube C and through tube E to the exit port F. This long pathway between the leak location and atmosphere was found necessary to eliminate back diffusion of air into the mass spectrometer. For operation of the mass spectrometer without the gas chromatograph, the leak tube is sealed off by screwed plugs at A and F and by pumping to fore vacuum via valve G.

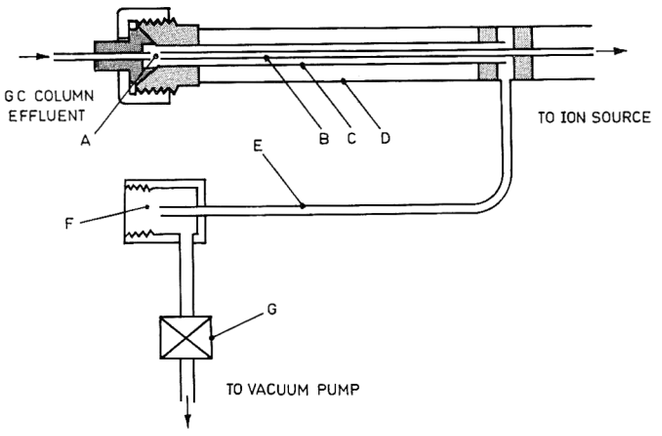

Fig. 1. GC-MS Connecting system (not to scale). Tube B: MS Leak tube 0.035 in O.D.; Tube C: 0.056 in O.D., 0.042 in I.D., length 4 in.; Tube D: 0.250 in O.D., 0.170 in I.D.; Tube E: 0.062 in O.D., 0.030 in I.D.

This avoids the need for a valve directly in the line from the gas chromatograph.

The surplus effluent may be collected at F, thus providing material for further examination by other techniques. This procedure has the merit that it enables the retention value, mass spectrum and IR spectrum to be obtained on material from the identical gas chromatographic peak.

The mass spectra were ratio-recorded (Brunnée *et al.*, 1963) using a simple system devised by Kennett (1967). Ratio recording involves registering the individual mass ion currents as ratios of the total ion current, thus eliminating the spectral distortion caused by the changes in concentration which occur during the scanning of a gas chromatographic peak. Ratio recording also allows the use of slower scan rates, thereby increasing the overall sensitivity and accuracy. A scan rate of 6 sec per mass octave was employed. Spectra were recorded on a 7-channel recording oscillograph ("Ultralette," ABEM, Stockholm). The ion source temperature was 250°C.

Little or no interference to the spectra was caused by phase bleed from the columns. This was attributed to the low bleed rate from the well-conditioned FFAP columns operated at moderate temperatures, coupled with the fact that individual constituents, for the most part, had been concentrated by the chromatographic fractionation of the flavor extracts.

Infra-red spectra

Samples of most of the alcohols introduced to the mass spectrometer were condensed from the effluent at exit port F. IR spectra were recorded either as a film or in solution depending on the sample size, this being previously judged from peak dimensions.

For examination as a film, the constituent was condensed in a glass melting point tube (9 cm × 1 mm, drawn down to about 0.2 mm at the outlet), attached by Teflon tubing at F and cooled by powdered carbon dioxide. The tube was then sealed by a fine flame at both ends and the condensate centrifuged into the tip. The broad end and then the extreme tip were cut off. The liquid sample was transferred by capillary action to the center of a rock salt plate (15 mm square) on which the film was contained within an area 4 mm × 1 mm isolated by an etched groove. The cell was completed by an aluminum foil washer (0.008 mm) to control film thickness, an unetched rock salt plate and an aluminum mask containing an aperture which aligned with the film area. The cell was lightly clamped in a stainless-steel holder which fitted a refractory beam condenser (RIIC). This simple laboratory-constructed cell was found suitable for liquids boiling above about 80°C and gave satisfactory spectra with samples as small as 0.025 μl.

Constituents of smaller volume than above were condensed in hypodermic needles according to the technique of Edwards *et al.* (1965), but using finer needles (22 gauge) as suggested by Edwards (1966, personal communication). With ends square cut, these needles snugly fitted the tapered filling hole of the RIIC MC3 microscope cell and it was thus possible to wash the condensate directly into the cell with 1–1.5 μl of carbon tetrachloride. The technique has given variable recoveries but nevertheless has enabled sensible information about the stronger absorption bands to be obtained from a few micrograms.

Reference compounds and spectra

The majority of alcohols were obtained from commercial supply houses and a few from other laboratories. The *trans* C_5, C_7 and C_8 alk-2-en-1-ols were synthesized. The IR and mass spectra of the reference alcohols were recorded after resolution on Columns A or B, using the same conditions as were employed for the pea alcohols. Where possible, their authenticity was verified by comparison of their mass spectra with API and ASTM reference spectra and with published spectra (Cornu *et al.*, 1966; Honkanen *et al.*, 1963).

RESULTS

TWENTY-TWO ALCOHOLS have been identified (Table 1). Positive identification was based primarily on the matching of their mass spectra with those of authentic compounds. In all cases this was supported by the agreement of gas chromatographic retention values.

The IR spectral evidence is summarized in Table 1. Only in a few cases were the spectra too weak to yield worthwhile information. Even where fragmentary spectra were obtained they still provided the essential evidence for the presence of strongly absorbing groups, e.g., hydroxyl, methyl and methylene C-H bonds, vinyl and *trans* unsaturation. This last named permitted conclusive differentation of the *cis* and *trans* isomers of the alk-2-en-1-ols and the alk-3-en-1-ols, which, although adequately resolved on polar columns, have mass spectra too alike to allow confident distinction (Honkanen *et al.*, 1963). The absence of *trans* absorption is indirectly indicative of a *cis* isomer.

The 2- and 3-methylbutan-1-ols were not resolved on FFAP but their mixed mass spectra clearly indicated their presence in a ratio 1:2. Confirmation of their presence and relative concentration was provided by their complete resolution on Carbowax 200.

On the basis of weak mass spectra or spectra from incompletely resolved peaks, several additional alcohols have been tentatively identified. These include *trans* hex-2-en-1-ol, *cis* and *trans* hept-3-en-1-ol and nonan-1-ol. In addi-

Table 1. Alcohols identified in unblanched green peas.

	M.S. Evidence[2]	I.R. Evidence	G.C. rentention time on FFAP relative to hexan-1-ol	Approx. amount relative to hexan-1-ol[3]
Methanol[1]	+	—	0.26	1000
Ethanol[1]	+	—	0.27	13000
Propan-1-ol[1]	+	Complete spectrum	0.34	0.2
Butan-1-ol[1]	+	Primary OH, CH_3, CH_2	0.46	0.02
Pentan-1-ol[1]	+	Primary OH, CH_3, CH_2	0.67	2
Hexan-1-ol[1]	+	Complete spectrum	1.00	100
Heptan-1-ol	+	Primary OH, CH_3, CH_2	1.53	0.5
Octan-1-ol	+	Primary OH, CH_3, CH_2	2.40	0.3
2-Methylpropan-1-ol[1]	+	Primary OH, isopropyl, CH_3, CH_2	0.39	0.08
2-Methylbutan-1-ol[1]	Mixed Spectra	Primary OH, isopropyl, CH_3, CH_2	0.58	2
3-Methylbutan-1-ol[1]	Mixed Spectra	Primary OH, isopropyl, CH_3, CH_2	0.58	4
Butan-2-ol	+	Spectrum too weak	0.25	
Pentan-3-ol	+	Spectrum too weak	0.41	
Pent-1-en-3-ol	+	Complete spectrum	0.49	1
Oct-1-en-3-ol	+	OH, vinyl	1.53	
Cis Pent-2-en-1-ol	+	Isolated—CH=CH—(3010), no *trans* unsatn.	0.87	
Trans Pent-2-en-1-ol	+	Spectrum too weak	0.84	
Cis Hex-3-en-1-ol[1]	+	Complete spectrum	1.18	17
Trans Hex-3-en-1-ol	+	Primary OH, *trans* unsatn.	1.04	
Trans Hept-2-en-1-ol	+	OH, *trans* unsatn.	2.00	0.4
Trans Oct-2-en-1-ol	+	OH, *trans* unsatn.	3.15	0.2
Cis Non-3-en-1-ol	+	Primary OH, isolated—CH=CH—, no *trans* unsatn.	4.00	0.2

[1] Previously identified by Ralls *et al.* (1965) in the volatiles from a commercial blancher. Hex-3-en-1-ol identified by them is presumed to have been *cis*.

[2] + denotes that the mass spectrum closely matched that of an authentic specimen.

[3] Calculated from peak areas from chromatograms of the oily concentrate. Methanol and ethanol values were estimated from chromatograms of the unconcentrated distillate. Where not specified, amounts were small but no estimate was possible.

tion trace amounts of three octenols are indicated, their short retention values suggesting that they are branched-chain.

DISCUSSION

AS INDICATED IN TABLE 1, ten of the 22 alcohols identified in this investigation were found by Ralls *et al.* (1965) in the vapor above peas in a commercial blancher. The remainder have not been reported previously as constituents of green peas.

An interesting feature of the present work is the identification of nine unsaturated alcohols of which only one has been previously found in peas. Several others are also present but, as yet, these have not been isolated in amounts sufficient for complete identification. Among the latter is *trans* hex-2-en-1-ol, which occurs widely in plant tissue and was expected to be one of the more abundant alcohols in green peas.

Hex-3-en-1-ol has long been recognized as a strong source of green leaf odor (Moncrieff, 1951) and oct-1-en-3-ol has been shown by Stark *et al.* (1964) to produce a "mushroom" odor in dairy products. The unsaturated alcohols may thus play an important role in pea flavor. To assess this possibility, the C_5 to C_9 *cis* and *trans* isomers of the alk-3-en-1-ols and the alk-2-en-1-ols are being synthesized.

No comparable evidence has been reported for the organoleptic importance of the saturated alcohols and it seems unlikely that ethanol and methanol, despite their abundance, would contribute notably to the flavor of peas. However, the higher molecular weight saturated alcohols, some of which occur in significant amounts, e.g., hexan-1-ol, could have some influence on flavor.

The relative concentrations of the alcohols in the extracts (Table 1), although probably indicative, cannot be regarded as an accurate guide to their relative concentrations in the raw material, as no reliable evidence has been obtained on the efficiency of extraction of the individual components.

Since the present investigations have been confined to unblanched peas it is uncertain whether the identified alcohols are products of abnormal metabolism subsequent to harvesting or whether they occur as normal constituents. Therefore, before investigating the significance of the alcohols to pea flavor, the identification of the nonalcoholic volatiles in unblanched peas will be completed, together with a study of the volatiles of fresh peas.

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R. H. WALTER [a] **and I. S. FAGERSON**
Department of Food Science and Technology
University of Massachusetts, Amherst, Massachusetts 01002

Volatile Compounds from Heated Glucose

SUMMARY—Volatiles from anhydrous glucose heated in air at 250°C for 30 min were collected in a trap maintained at the temperature of solid carbon dioxide. A concentrated ether extract of the distillate was shown by gas chromatography to contain at least 100 compounds. Among those identified and heretofore unreported from heated glucose were the gamma lactone of 4-hydroxy-2-pentenoic acid, 1-(2'-furyl)-propane-1,2-dione (acetylfuroyl), 3-methylcyclopentane-1,2-dione, phenol and methylfuroic acid.

INTRODUCTION

A NUMBER OF PUBLICATIONS have recently appeared on the application of gas chromatography to the analysis of volatile products from the thermal decomposition of carbohydrates and closely related substances: Bailey *et al.* (1962), Bryce *et al.* (1963), Doerr *et al.* (1966), Fiddler *et al.* (1966), Gianturco *et al.* (1963, 1964), Greenwood *et al.* (1961), Graham (1966), Heyns *et al.* (1966a), Sugisawa (1966), and Tai *et al.* (1964). These studies indicate the presence of a wide variety of compounds, but generally only the low boiling classes have received major attention. Compounds of intermediate volatility have not been as extensively studied despite some indication that they may be more important contributors to flavor.

Since glucose was present in substantial amounts in the materials previously studied, it was felt that heating glucose itself in an isolated system would give valuable information on the origin of the volatile flavor compounds.

Among the variables that may affect the composition of volatiles from glucose are heating time, temperature, and the presence of oxygen. On the basis of achieving an adequate collection of volatiles in a reasonable time, a temperature roughly intermediate between 150°C (Sugisawa, 1966) and 300°C (Heyns *et al.*, 1966a) and a heating period of 30 min were chosen. The heating was carried out in atmospheres of both air and nitrogen.

EXPERIMENTAL

Heating and distillation

Two g of anhydrous glucose (Baker analyzed reagent) were placed in a 50 ml (₹ 45/50) thre-neck distillation flask in which a coarse capillary and a thermometer were fixed by means of Teflon adapters (Fig. 1). A side-arm of the flask led into a trap which was surrounded by dry crushed ice. The system was connected to a water aspirator or to a cylinder of "pre-purified grade" N, to obtain either the air or N atmosphere over the heated glucose. Heat was provided by an electric heating mantle which was thermostatted by a Jelrus automatic controller (manufactured by the Jelrus Co., Inc., New York, N. Y.) to maintain an internal flask temperature of approximately 250°C. The total distillation time was 30 min.

[a] Present address: International Flavor and Fragrances, Union Beach, N. J.

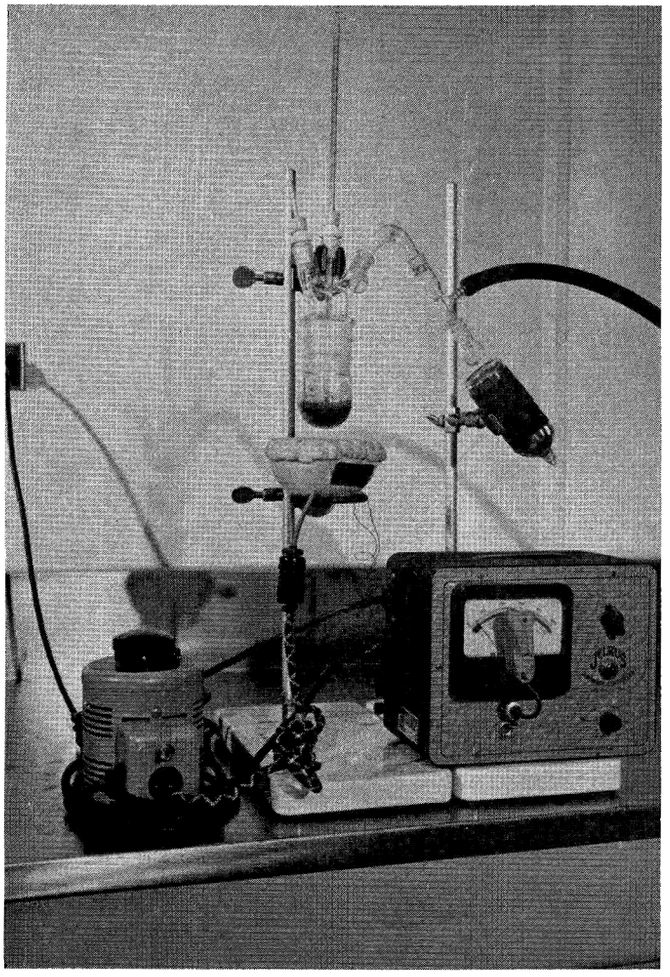

Fig. 1. Distillation system for the collection of volatiles.

Extraction and concentration

The frozen distillate, usually less than 1 ml, was diluted to about 3 ml with distilled water, transferred to a 150 × 16 mm test tube, enough anhydrous Na_2SO_4 added to saturate the solution, and extracted with an equal volume of redistilled, anhydrous ether. The ether phase was then pipetted into a 5 ml beaker, and the ether slowly evaporated under a stream of N to about one-third the original volume, after which, it was transferred with a pipet to a 3 ml conical bottom, glass-stoppered centrifuge tube for further evaporation under N. The final concentration was arbitrary, but evaporation was terminated when there was a noticeable increase in viscosity of the ether extract. The final volume was about 0.5 ml. This concentrate had a refrigerated storage life of 7–10 days at 3°C, after which, it tended to form a precipitate. Stability could be increased somewhat by redilution with anhydrous ether prior to storage.

When it was desired to eliminate the masking effect of the ether solvent on compounds of very short retention times during subsequent gas chromatography, the undiluted distillate was transferred directly to the centrifuge tube and diluted with 2.0–2.5 ml of a saturated Na_2SO_4 solution, then centrifuged at 4000 rpm until an oily, brown ring separated at the surface. From this layer, 0.5 μl samples were withdrawn for direct injection into the gas chromatograph-mass spectrometer unit.

Analysis

One to five micro-liter samples of the concentrated ether extract were taken for gas chromatography. Mass spectra were obtained, using a coupled gas chromatograph (Perkin-Elmer Model 226)—mass spectrometer (Hitachi-Perkin-Elmer Model RMU-6A) unit with a capillary column, 200 ft long, 0.02 in. ID, and coated with a mixture of diethyleneglycolsuccinate and H_3PO_4 (2%). The column effluent was split with equal portions being directed to the flame ionization detector, and to the mass spectrometer, the latter via a Watson-iBemann, helium separator (Watson *et al.*, 1965). The operating parameters were as follows:

Gas chromatograph:	
flow rate	Helium carrier gas at 15 ml/min.
program	30°C for 5 min, then 2°C/min to 160°C
injection block temperature	275°C
Mass spectrometer:	
inlet temperature	200°C
ion source pressure	4×10^{-6} mm
ion source temperature	250°C
acceleration voltage	2.5 Kv
target current	60uA
electron multiplier voltage	2.5 Kv
scan speed	12 sec.(m/e 12-400)
exit slit	0.15 mm

Trapping for infrared analysis was attempted by the method of Edwards *et al.* (1965), from a Perkin-Elmer 810 gas chromatograph with a 6 ft long, ⅛-in. OD column packed with 10% polypropyleneglycol on 60/80-mesh Chromosorb W. The outlet temperature was kept at 250°C, and the injection block temperature at 275°C. The column was held at 65°C for 5 min, and then ballistically programmed to 200°C where it remained until completion of the analysis. Twenty % of the carrier gas, N was diverted to the flame ionization detector, and 80% to the outlet.

The presence of formaldehyde and CO_2 was established in separate experiments by bubbling the sweep gas from the heated glucose into a solution of 1% chromotropic acid in concentrated H_2SO_4 (Frisell *et al.*, 1958) and 0.05% $Ba(OH)_2$ solution respectively. The development of a purple color in the former and a precipitate in the latter was taken as evidence of the presence of these compounds.

RESULTS AND DISCUSSION

On the basis of comparative gas chromatography, there was no evidence that the heating atmosphere significantly influenced the composition of the collected volatiles.

Since primary emphasis in this study was placed on compounds of intermediate volatility, no special attempt

Table 1. Compounds identified in the volatile fraction of heated glucose.

G.C. No.	Compounds	Method of identification I.R.	G.C.	M.S.	Remarks
	CO_2				See experimental
	Formaldehyde				See experimental
1	Furan		++	++	
2	2-Methylfuran		++	++	
2	Butadione (Diacetyl)		++	++	
4	Acetic acid		++	++	
6	2-n-Propylfuran			+	Heyns *et al.* (1966b)
8	Furfural (2-furaldehyde)	++	++	++	
9	2-Acetylfuran		++	++	
12	3-Furaldehyde			+	Heyns *et al.* (1966b)
15	5-Methyl-2-furaldehyde		++	++	
16	5-Methyl-2-acetylfuran			+	
24	4-Hydroxy-2-pentenoic acid, gamma lactone		++	++	
27	1-(2'-furyl)-propane-1,2-dione (acetylfuroyl)		++	++	
29	3-Methylcyclopentane-1,2-dione		++	++	
31	Phenol		++	++	
42	Methylfuroic acid (one of various positional isomers.)				By mass spectrum interpretation only

++ Agreement with authentic compounds.
* Mass spectral data only.

was made to conserve the more volatile components during distillation. Many of these have recently been reported by Qua *et al.* (1964), Heyns *et al.* (1966a), and Sugisawa (1966).

At least 100 compounds were recovered by the method of distillation and concentration employed, as shown by a typical gas chromatogram (Fig. 2). Table 1 lists the compounds identified and their method of identification.

Identifications were made by first obtaining mass spectra of the eluting peaks to ascertain probable structures which were subsequently confirmed or rejected on the basis of comparison with mass spectra of authentic samples. Confirmation of a specific structure was then determined by comparison of gas chromatographic retention times with authentic samples.

Except for furfural, attempts to obtain useful infrared spectra from the packed column were unsuccessful, possibly because of the low concentrations involved, and the low efficiency of recovery from the injected sample.

Of the compounds identified, the gamma lactone of 4-hydroxy-2-pentenoic acid 1-(2'-furyl)-propane-1,2-dione ,acetylfuroyl), 3-methylcyclopentane-1,2-dione, phenol and methylfuroic acid are not heretofore known to have been reported as arising from heated glucose. The mass spectra of these compounds are presented in Fig. 3.

The existence of phenol as a volatile compound from heated glucose is observed with special interest, due to its known toxic properties. The presence of the gamma lactone of 4-hydroxy-2-pentenoic acid is noteworthy, because of the implication of unsaturated gamma lactones in anti-

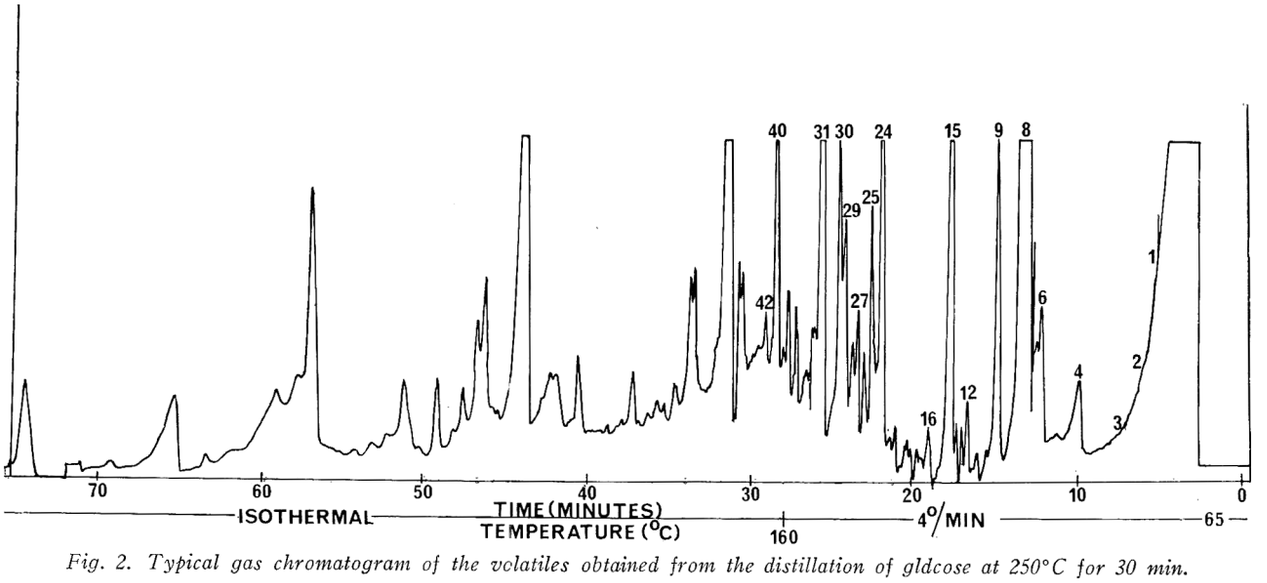

Fig. 2. Typical gas chromatogram of the volatiles obtained from the distillation of gldcose at 250°C for 30 min.

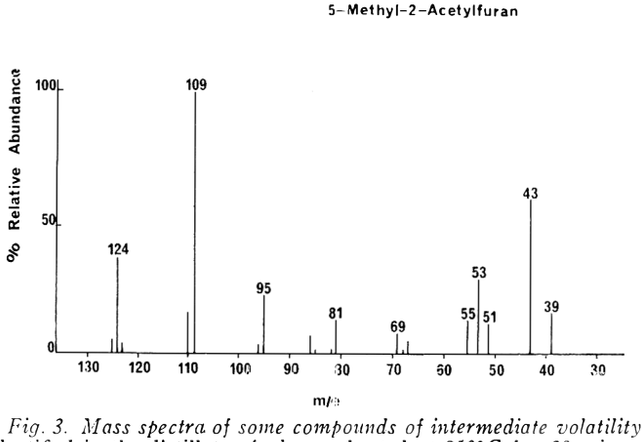

Fig. 3. Mass spectra of some compounds of intermediate volatility identified in the distillate of glucose heated at 250°C for 30 min.

biosis (Oxford 1945). Also, an enolic isomer of 3-methylcyclopentane-1,2-dione is reported as being used to impart a maple flavor and as a flavor potentiator by Filipic *et al.* (1965).

The mechanism of formation of 4-, 5-, and 6-carbon furan structures from monosaccharides has already been proposed Newth (1951), Anet (1964), and Byrne *et al.* (1966), but the presence of 7-carbon furan compounds in the volatiles from heated glucose (propylfuran, methylacelytfuran and 1-(2′-furyl)-propane-1,2-dione leads to the suspicion that secondary reactions of primary decomposition products also influence the vapor phase composition. For example, acetic acid and furfural could conceivably react to form 1-(2′-furyl)-propane-1,2-dione. In previous works, this compound was obtained only as an oxidative product of 2,furylacetone (Cosgrove *et al.*, 1952) and one component of coffee volatiles (Gianturco *et al.*, 1964). Such a compound, however, could also originate from a polymer fragmentation process.

A common source of benzenoid and alicyclic compounds is the pyrolysis of carbonaceous matter (bituminous coal) at very high temperatures (1000–3000°C), in the absence of air. The presence of phenol and 3-methylcyclopentane-1, 2-dione at 250°C may therefore indicate a common reaction path, but the details of the mechanism of formation of these structures from heated glucose has yet to be elucidated.

Most of the compounds identified have a unique, mild aroma none of which could be described as that of caramel. It may therefore be that the caramel aroma itself arises from the mixture of volatiles present.

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RICHARD A. BERNHARD
Department of Food Science and Technology, University of California, Davis, California 95616

Comparative Distribution of Volatile Aliphatic Disulfides Derived from Fresh and Dehydrated Onions

SUMMARY—Gas chromatographic determination of the volatiles from fresh "Sunspice" onions revealed that the principal disulfide present is di-*n*-propyl, followed in descending order of concentration by *n*-propyl allyl, methyl-*n*-propyl, methyl allyl, dimethyl, and diallyl. In dehydrated onions this order is markedly altered. Methyl-*n*-propyl is the principal disulfide, followed by dimethyl, methyl allyl, di-*n*-propyl, *n*-propyl allyl, and diallyl. Quantitative estimates of the disulfide content of 53 lots of fresh onions were made and compared with analyses of dehydrated onions from comparable lot numbers. Loss of measured volatiles averaged 98%, while loss of disulfides was greater than 89%. The relationship of disulfides to onion flavor and a pungency ranking system based on these analyses are discussed.

INTRODUCTION

ON PASSING ANY FOOD processing plant, even the casual observer can detect strong characteristic product odors. These odors, whether they come from an onion processing plant, bakery, or coffee roaster, can often be noticed 10 to 20 miles away depending upon prevailing winds and climatic conditions.

This study was initiated to determine the nature, and to establish the quantity, of volatile losses during the processing and dehydration of onions. An attempt was also made to relate the volatile losses through processing to the aroma and flavor of fresh and dehydrated onions.

Although the measured total disulfide differences between fresh and dehydrated onions was greater than 89%, it is quite obvious that dehydrated onions are sufficiently full-flavored that they are now used in place of raw onions by most food processors.

Loss of components from onions on heating or drying has been studied extensively by a number of researchers. Titov *et al.* (1964) established that on termination of sublimation drying of onions, ascorbic acid content was reduced by 50%; and that during heat drying, ascorbic acid destruction was even greater. Copeman *et al.* (1947) reported that only small losses of nitrogen and sulfur occur because of volatilization during drying. They came to this conclusion by measuring the total sulfur and nitrogen content of the onions before and after dehydration.

Studying chemical changes in onions produced by boiling, Yamanishi and Orioka (1955) determined the quantity of *n*-propyl allyl disulfide lost. They concluded that while *n*-propyl allyl disulfide is lost on boiling, the quantity of *n*-propanthiol increases. The suggestion was advanced that *n*-propanthiol and di-*n*-propyl disulfide are produced from *n*-propyl allyl disulfide during this process. Taste tests made by these authors revealed that *n*-propanthiol is some 50 to 70 times as sweet as sucrose. Thus they concluded that the sweet taste developed in boiled onions was due, at least in part, to the development of the thiol.

Unpublished work by Haagen-Smit indicated that after "a few minutes" little or no aroma could be detected coming from onions in a drying oven, the conclusion being that there was little volatile loss actually associated with the act of drying (Stephenson, 1949).

Analysis of onion flavor is complicated by the fact, as stated by Stephenson (1949), that, "Onions available in any locality throughout the calendar year may vary as much as 300 to 400% in flavor and solids." Kohman (1952) also pointed out that variations in chemical composition as great as 7-fold may occur when comparing top and bottom or right and left halves of a single onion bulb.

Indeed one must conclude that analysis of flavor and aroma, whether it be from fresh, boiled, or dried onions, is extremely complex. Certainly many aspects of it are not now well understood.

EXPERIMENTAL

Apparatus

The gas chromatographic equipment used in this study was an Aerograph model 600-C HI-FI equipped with flame ionization detection. All columns were constructed of ⅛ in o.d. stainless steel tubing. Columns were 10 ft in length, packed with a mixture of 5% (w/w) Carbowax 20M on acid-washed firebrick 100/120 mesh. Column temperature was maintained at 90°, injector temperature at 145°, and detector temperature at 90°. Nitrogen was used as the carrier gas and the flow rate was 25 ml/min. Hydrogen flow rate was also 25 ml/min; air flow rate was 300 ml/min. For readout, a 1-mv potentiometric recorder equipped with a variable span input attenuator was employed. For the analyses of fresh onions, a span of 5 mv and a chart speed of 20 in./hr was used. Since the quantities of volatiles from dried onions were substantially less than those from the fresh, a span of 2 mv was used, and the chart speed increased to 40 in./hr to achieve greater accuracy in the integration of the smaller peaks. For area counts, these span and chart speed differences were normalized so areas for both fresh and dehydrated onions are reported in the same units and under the same parameters of operation.

Samples

Fresh onion samples were obtained from the experimental test plots. These onions were *Allium cepa* of the group belonging to the common onion and the cultivar "Sunspice," a strain of Improved Southport White Globe. They were grown in various parts of California and harvested at different dates during the year of 1966.

Procedures

Preparation of onions for drying was in conformity with commercial practice. Dehydration of samples was accomplished using a Despatch gas-electric drying oven (Despatch Oven Co., Minneapolis, Minn., Style V-29). Drying temperature was 130 to 135°F and the onions were dried to 5 to 5½% moisture content. Soluble solids were determined using a hand refractometer calibrated in percent solids.

Fresh and dried onion samples were examined chromatographically as follows: Fresh samples were sliced and chopped using a razor blade (Saghir *et al.*, 1964). Ten g of chopped tissue were placed in a 30-ml screw-capped glass vial. Prior to introducing the sample, a small hole was drilled in the cap and a circular Teflon septum placed inside the cap. Samples could be withdrawn from the vial through the septum without loss of vapor. The vial was held at 40° in a thermostatically controlled circulating air oven for 30 min before withdrawal of 1.0 ml of vapor for analysis. The 30-min incubation period is necessary for the maximum development of the disulfides examined, and this aspect is discussed fully by Saghir *et al.* (1964).

Dehydrated samples were examined in much the same fashion except that no chopping was necessary. Dried material was crushed to commercial flake size. Nine g of dried onions were placed in a 30-ml vial and 5 ml of distilled water added. The cap was screwed in place and the contents shaken to achieve mixing and start rehydration. The samples were incubated for the usual 30 min at 40° prior to chromatographic examination.

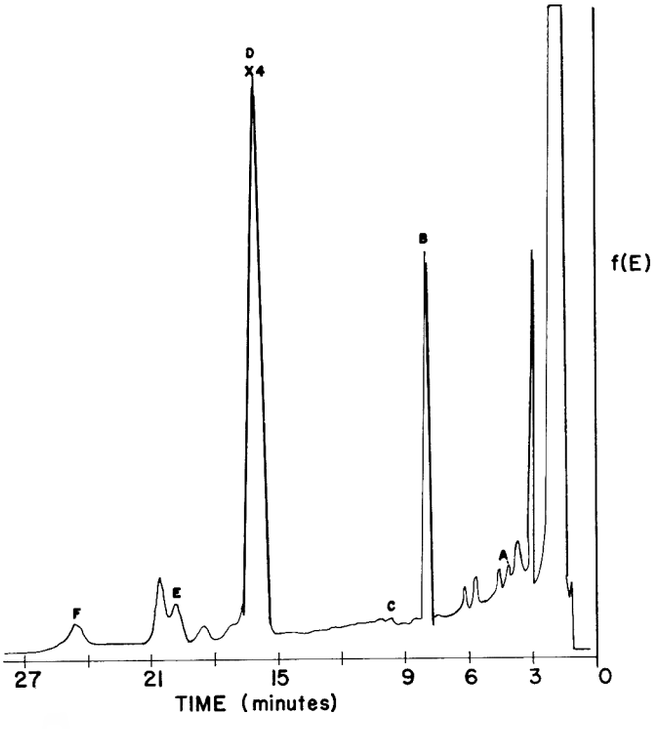

Fig. 1. Gas chromatogram of vapor of fresh A. cepa *L., "Sunspice." See text for conditions of operation. A—dimethyl disulfide; B—methyl-*n*-propyl disulfide; C—methyl allyl disulfide; D—di-*n*-propyl disulfide; E—*n*-propyl allyl disulfide; and F— diallyl disulfide.*

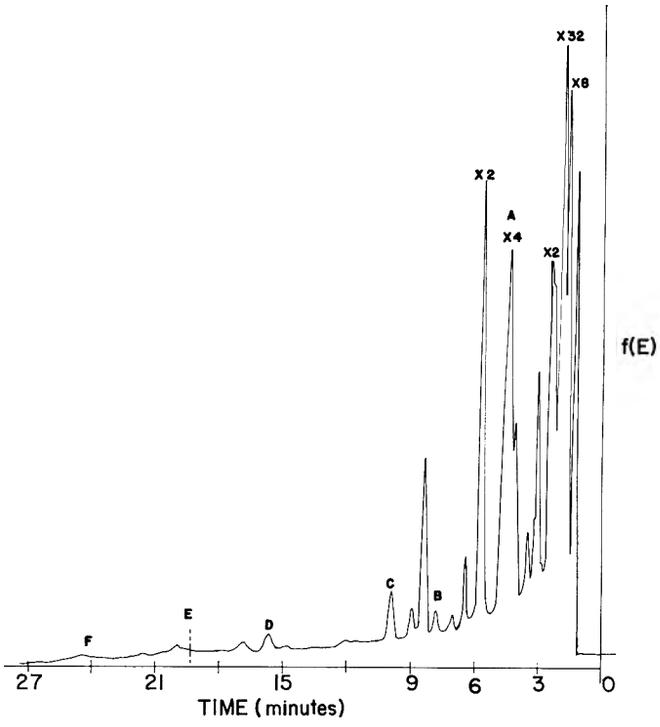

Fig. 2. Gas chromatogram of vapor of dried A. cepa *L., "Sunspice." See text for conditions of operation. A—dimethyl disulfide; B—methyl-*n*-propyl disulfide; C—methyl allyl disulfide; D—di-*n*-propyl disulfide; E—*n*-propyl allyl disulfide; and F—diallyl disulfide.*

The ratio of 9 g of dried sample to 5 ml of water was arrived at by an empirical approach. Various rehydration ratios were tried, and this ratio gave the greatest production of volatiles.

Identification of the disulfides was accomplished as described previously (Saghir *et al.*, 1964).

RESULTS AND DISCUSSION

The chromatograms of vapors of fresh "Sunspice" onion (Fig. 1) and of dried "Sunspice" onion (Fig. 2) are typical of those obtained from all of the onions analyzed. These chromatograms indicate the size of the peaks obtained with 1 ml of vapor, the position of the various disulfides, and the presence of unidentified peaks.

The amounts of disulfides present in the samples of vapor have been estimated from chromatograms by measurement of peak areas (Table 1). The values are expressed as a percent of the total area of all disulfide peaks identified on each chromatogram. With sample number 501, for example, assuming that the six peaks include most of the disulfide components of the vapor, di-*n*-propyl disulfide constitutes slightly over half of the total area of disulfides in the vapor. These data then present a measure of the relative amounts of these compounds present in each sample.

The actual areas under the disulfide peaks are presented in Table 2. These data permit comparison of the amounts of disulfides between various samples and the changes in disulfide concentration after drying.

Lot numbers were assigned on the basis of breeding

Table 1. Areas of peaks[1] representing disulfides in fresh and dried onion samples as percentage of total disulfides measured.

Lot No.	Me_2S_2		$MePrS_2$		$MeAlS_2$		Pr_2S_2		$PrAlS_2$		Al_2S_2		Fresh % Soluble solids
	Fresh	Dried	Fresh	Dried	Fresh	Dried	Fresh	Dried	Fresh	Dried	Fresh	Dried	
501	2	34	12	22	7	10	59	6	20	10	b[2]	18	17.6
629	3	37	11	32	6	16	66	9	14	4	b	2	18.0
633	2	33	10	33	9	16	63	6	16	10	b	2	13.9
689	b	48	5	24	4	13	68	7	23	5	b	3	16.2
701	2	41	10	31	5	16	71	7	12	5	b	b	15.7
773	4	29	8	46	5	13	64	12	19	b	b	b	10.7
1035	b	26	2	41	5	13	74	3	19	11	b	6	8.8
1037	b	34	4	43	6	12	80	11	10	b	b	b	10.1
1053	b	33	9	39	9	15	65	6	17	6	b	1	17.0
1343	1	48	9	27	6	16	62	9	22	b	b	b	16.0
1345	1	32	8	39	13	18	71	11	7	b	b	b	14.4
1361	2	41	12	33	5	19	69	2	12	3	b	2	14.8
1363	b	43	7	36	6	14	74	7	13	b	b	b	13.0
1368	b	27	7	44	5	29	72	b	16	b	b	b	16.2
1431	b	43	5	31	8	15	77	4	10	4	b	3	13.4
1433	b	38	6	31	8	14	70	12	16	3	b	2	15.0
2057	2	26	10	48	3	12	71	14	14	b	b	b	13.6
2077	b	19	6	52	5	9	76	20	13	b	b	b	14.2
2117	b	46	4	31	9	13	68	5	19	3	b	2	14.2
2139	3	32	6	43	4	19	68	6	19	b	b	b	14.7
2327	b	32	5	43	3	13	85	12	7	b	b	b	16.2
2365	b	43	8	31	7	13	67	10	18	4	b	b	16.4
2399	b	48	10	29	5	14	62	5	23	4	b	b	15.3
2425	b	42	7	34	5	14	78	10	10	b	b	b	16.4
2459	2	35	23	38	3	22	59	5	13	b	b	b	18.2
2595	b	40	6	34	8	12	74	9	12	5	b	b	17.3
2667	b	27	5	58	4	8	67	7	24	b	b	b	15.8
3001	b	36	7	41	4	14	80	9	9	b	b	b	16.2
3002	4	30	5	49	2	12	84	9	5	b	b	b	14.8
3003	b	23	4	44	5	18	88	15	3	b	b	b	16.6
3004	b	25	6	40	3	23	87	12	4	b	b	b	17.2
3006	5	29	9	44	4	13	77	14	5	b	b	b	12.4
3007	3	32	9	43	4	17	76	8	8	b	b	b	14.6
3008	2	33	10	32	3	17	75	18	10	b	b	b	15.8
3009	b	33	4	40	5	17	86	8	5	b	b	2	18.0
3010	b	34	5	43	8	13	80	11	6	b	1	b	18.5
3012	b	27	6	45	b	16	77	12	17	b	b	b	17.2
3013	b	26	14	48	5	14	66	12	15	b	b	b	19.2
3014	b	28	8	37	4	12	79	23	9	b	b	b	14.8
3015	b	25	8	39	6	17	78	19	8	b	b	b	17.8
3016	b	28	2	44	1	16	92	12	5	b	b	b	15.8
3017	b	20	8	40	2	17	77	20	13	b	b	3	16.2
3018	3	33	6	43	4	14	77	10	10	b	b	b	16.3
3019	b	30	6	46	2	16	85	8	7	b	b	b	17.8
3021	b	37	4	40	6	12	84	6	6	2	b	3	13.5
3034	2	36	8	46	3	18	72	b	15	b	b	b	18.6
3035	4	22	5	45	6	18	76	15	9	b	b	b	17.8
3051	b	23	9	36	3	15	65	8	23	12	b	6	19.6
3054	b	26	13	38	4	14	70	9	13	8	b	5	15.3
3059	b	26	6	37	7	13	75	12	12	8	b	4	18.8
3080	4	35	21	40	4	16	63	9	8	b	b	b	14.8
3081	4	33	6	50	1	12	74	5	15	b	b	b	18.4
6265	5	52	8	24	3	15	79	2	5	2	b	5	11.1

[1] Assuming peak areas are triangular, area = height × width of base/2. Where peaks are attenuated, area is multiplied by attenuation factor.
[2] Not detectable.

Table 2. Chromatographic peak areas[1] of disulfides from fresh and dried onion samples.

Lot No.	Me_2S_2		$MePrS_2$		$MeAlS_2$		Pr_2S_2		$PrAlS_2$		Al_2S_2		Total area	
	Fresh	Dried	Fresh	Dried	Fresh	Dried	Fresh	Dried	Fresh	Dried	Fresh	Dried	Fresh	Dried
501	740	925	3735	585	2000	280	17795	146	6025	281	b[2]	487	30,295	2,704
629	885	852	3735	726	2130	364	21800	196	4570	98	b	39	32,960	2,275
633	420	406	2280	418	2035	200	14695	73	3725	131	b	27	23,155	1,255
689	b	957	960	470	795	257	12370	145	4055	95	b	50	18,180	1,974
701	515	825	3155	598	1390	310	21290	130	3545	90	b	b	29,895	1,953
773	1510	474	2800	760	1770	204	21350	200	6390	b	b	b	33,820	1,638
1035	b	441	650	672	1335	220	21420	55	5385	185	b	102	28,970	1,675
1037	b	848	1450	1088	2190	320	30675	272	3880	b	b	b	38,195	2,528
1053	b	541	2345	634	2540	243	17995	90	4620	104	b	21	27,500	1,633
1343	315	680	2875	382	1960	230	19500	128	7005	b	b	b	31,655	1,420
1345	890	680	4325	836	7430	396	40685	240	4295	b	b	b	57,625	2,152
1361	850	409	4975	337	2010	193	27550	23	4990	33	b	23	40,375	1,018
1363	b	1376	1880	1152	1645	448	20840	256	3505	b	b	b	27,870	3,232
1368	b	608	1570	1008	1080	672	16435	b	3545	b	b	b	22,630	2,288
1431	b	728	1450	517	2165	260	21460	68	2835	73	b	46	27,910	1,692
1433	b	1150	1560	922	1955	425	17580	355	3880	103	b	53	24,975	3,008
2057	990	792	5895	1424	1800	368	39740	408	8130	b	b	b	56,555	2,992
2077	b	456	2310	1264	2180	216	30095	488	5225	b	b	b	39,810	2,424
2117	b	865	875	595	2075	239	15370	98	4260	51	b	45	22,580	1,893
2139	1210	520	2410	724	1620	324	24920	104	6985	b	b	b	37,145	1,672
2327	b	358	3865	486	2880	151	70700	125	6165	b	b	b	83,610	1,120
2365	b	902	1785	661	1625	266	15025	201	4025	90	b	b	22,460	2,110
2399	b	1236	2885	746	1430	353	18065	139	6940	102	b	b	29,320	2,576
2425	b	752	3010	608	2270	252	32310	174	4150	b	b	b	41,740	1,786
2459	910	1110	12230	1212	1615	684	31880	150	6955	b	b	b	53,590	3,146
2595	b	843	1590	705	2080	235	18585	194	2960	105	b	b	25,215	2,082
2667	b	518	1720	1114	1420	163	23820	139	8585	b	b	b	35,545	1,934
3001	b	650	2930	742	1645	252	36025	174	4150	b	b	b	44,750	1,818
3002	2460	752	2960	1204	1215	288	46650	232	2825	b	b	b	56,110	2,476
3003	b	421	3730	821	4165	342	76160	280	2290	b	b	b	86,345	1,864
3004	b	624	4535	1019	2310	581	60490	302	3030	b	b	b	70,365	2,526
3006	2035	727	3630	1121	1670	325	31440	363	2060	b	b	b	40,835	2,536
3007	2010	588	7275	782	2845	312	61320	144	6480	b	b	b	79,880	1,826
3008	1165	896	6045	872	1730	432	44920	488	6190	b	b	b	60,050	2,688
3009	b	546	2860	664	4025	280	65370	124	4125	b	b	34	76,380	1,648
3010	b	752	3330	940	4685	280	49820	232	3665	b	545	b	62,045	2,204
3012	b	502	535	824	b	294	6860	222	1545	b	b	b	8,940	1,842
3013	b	300	4705	574	1695	172	21845	144	4890	b	b	b	33,135	1,190
3014	b	378	3605	508	1755	168	34505	312	3720	b	b	b	43,585	1,366
3015	b	676	2195	1062	1585	462	21850	504	2110	b	b	b	27,740	2,704
3016	b	396	1030	622	480	228	38375	176	2015	b	b	b	41,900	1,422
3017	b	536	3545	1050	905	441	36355	522	6135	b	b	89	46,940	2,638
3018	1465	612	2610	800	2080	264	36275	192	4725	b	b	b	47,155	1,868
3019	b	556	3185	836	1200	284	45465	152	3860	b	b	b	53,710	1,828
3021	b	486	1290	529	1975	153	28310	73	2100	23	b	36	33,675	1,300
3034	820	620	2875	800	1090	308	24715	b	5305	b	b	b	34,805	1,728
3035	1835	596	2685	1222	3030	501	38800	421	4475	b	b	b	50,825	2,740
3051	b	268	3380	424	1110	180	24220	98	8740	143	b	76	37,450	1,189
3054	b	466	5060	680	1495	252	27285	160	5120	152	b	73	38,960	1,788
3059	b	530	3090	762	3415	255	38095	251	6345	166	b	74	50,945	2,038
3080	2270	770	12010	906	2070	354	36225	212	4640	b	b	b	57,215	2,242
3081	2350	913	3450	1430	600	324	41040	133	8685	b	b	b	56,485	2,800
6265	1610	1204	2860	550	915	350	27320	40	1810	50	b	119	34,515	2,313

[1] Expressed in mm^2.
[2] Not detectable.

lines and hybrids (a cross between two or more breeding lines). Samples with the same lot number were grown in various parts of California, but came from the same seed lot. There are 53 lots presented in this study. Four replicate samples of each fresh onion lot and four of each dried onion lot were examined chromatographically.

The precision of the integration was limited by the fact that we were unable to measure accurately peak widths to better than ± 0.2 mm. Thus for example, on narrow peaks of 2 mm, reproducibility is ± 10% in estimation of base width. All of the peaks examined in this study had minimum base widths of 6 mm or more. Thus the precision of integration was approximately ± 3%. Since no two onion bulbs are precisely alike as to component concentration, reproducibility between bulb samples of the same lot number varied on the average ± 5%, but some few varied as much as ± 15%. The author chose the median of the four replicates (because of the small population) as being statistically most meaningful.

In order to verify that the dehydrated samples were comparable to commercially dried samples, 50 samples of commercial "Sunspice" dehydrated onion were examined chromatographically using the procedures outlined above. These samples gave chromatograms similar to those obtained from the samples prepared for this study.

Little is known about onion flavor, although investigations have been in progress since the late nineteenth century (Semmler, 1892). Members of the genus *Allium*—onions, garlic, leek, chive, etc.—possess strong, characteristic aromas and flavors not found in other vegetables. A remarkable property of this genus is that most members have no odor unless the plant tissue is cut or otherwise damaged in some fashion. Stoll *et al.* (1951) found that these characteristic volatiles are absent from intact tissues, and the volatiles are enzymatically produced when injury occurs.

Substrates for the production of these volatiles are known as alliins and are derivatives of the amino acid, cysteine. The alliins give rise through several reactions to the sulfur-containing volatiles. A typical alliin, S-allyl cysteine, sulfoxide, on treatment with the enzyme allinase yields an allicin, in this instance, diallyl thiosulfinate. Allicin, unlike the odorless alliin, is volatile and has a pleasant garlic-like odor. It is unstable, however, and allyl disulfide is produced as one of its breakdown products. Other alliins containing methyl and propyl radicals have been isolated, and these can then give rise to a number of disulfides (Virtanen *et al.*, 1959).

It has been advanced by several authors (Saghir *et al.*, 1964) that the allicins are responsible for the odors of freshly cut alliums, and that similar, but perhaps less-pleasant-smelling disulfides and trisulfides derived from various allicins also play an important role in *Allium* flavor. How rapidly the allicins break down in freshly prepared onions is still not known; thus the disulfides can conceivably contribute to odor and flavor too. This might be especially true in cooked or dehydrated onions. Even if the disulfides revealed by gas chromatography are artifacts of analysis, they are clearly related to onion aroma and flavor.

It should be noted that other factors such as sugars, total solids, crispness, and the lachrymator may have profound effects in the eating quality of onions too, depending especially on how the onions are used.

Six volatile aliphatic disulfides are known to be present in onions. They are: dimethyl disulfide (Me_2S_2); di-*n*-propyl disulfide (Pr_2S_2); diallyl disulfide (Al_2S_2); methyl-*n*-propyl disulfide ($MePrS_2$); methyl allyl disulfide ($MeAlS_2$); and *n*-propyl allyl disulfide ($PrAlS_2$) (Saghir *et al.*, 1964; Jacobsen *et al.*, 1964; Bernhard, 1966). Virtanen (private communication, 1967) has indicated that 1-propene analogs of the disulfides exist in some *Allium*, but to date the author has found no evidence of these compounds in the onions examined in this study.

Dimethyl disulfide has a strong cabbage-like odor; while di-*n*-propyl disulfide has the aroma typically associated with onions. Diallyl disulfide has a definite garlic-like aroma. The mixed disulfides have odors intermediate between those of the three symmetrical disulfides. The allyl and propyl radicals appear to dominate the aroma of the methyl component, and the author has noted that onions with a high percentage of methyl disulfide do not necessarily smell like cabbage. The methyl component can only be detected if it forms a very large portion (> 80%) of all of the disulfides present.

It is difficult to make accurate estimates of the kind and amount of allicins in chopped onions since they are participating in reactions that convert them to the more stable disulfides. The disulfides lend themselves to rapid qualitative and quantitative analyses by gas chromatography (Saghir *et al.*, 1964). Thus the author chose to examine the disulfides of onions and follow changes in kind and amount during processing. In this way also an index of aroma and flavor can be followed from the fresh to the dehydrated product.

The preponderant disulfide in fresh onions is *n*-propyl (Table 1). Its concentration exceeds those of all the others by a wide margin and undoubtedly determines to a considerable extent the character of the flavor and aroma of the fresh onion. *n*-Propyl allyl, methyl-*n*-propyl, methyl allyl, dimethyl, and diallyl disulfides follow in descending order of relative percentage. In dehydrated onions this order is changed quite markedly, methyl-*n*-propyl disulfide being present in greatest amounts. This is followed by dimethyl, methyl allyl, di-*n*-propyl, *n*-propyl allyl, and diallyl disulfides. On this basis alone one could expect a marked change in the flavor of the two onion forms. Fresh "Sunspice" onions have a rich, pungent aroma with slight overtones of garlic flavor; they have strong lachrymatory properties. The dehydrated onions show reduced lachrymatory effects, and have an aroma reminiscent of a mixture of di-*n*-propyl disulfide and a cabbage-like note. In some samples there were traces of a grass-like odor. Regardless of personal descriptions, weak and unreliable as they undoubtedly are, there are noticeable differences in both the flavors and aromas of fresh and dried onions of the same cultivar.

It may also be observed in Table 1 that the relative percentages show considerable variation from lot to lot. Data from the 212 determinations of disulfide content of fresh onions were fed into a computer programmed to present correlation information (Program: BMD02D, correlation

with transgeneration). Results indicated that there was a high degree of correlation between di-*n*- propyl disulfide concentration and total disulfide concentration (correlation = 0.9628). This seems fairly obvious from looking at the data (Table 2).

There was also correlation between methyl-*n*-propyl disulfide and di-*n*-propyl disulfide (correlation = 0.6331) and between *n*-propyl allyl disulfide and di-*n*-propyl disulfide (correlation = −0.7927).

These correlations suggested an interesting possibility; is there any correlation between the objective method of measuring disulfide distribution and the subjective methods of measuring onion pungency? From a commercial onion dehydration corporation, the author obtained "Sunspice" onions that were in the organization's opinion well-defined examples of strong, medium, and mild onions. These samples were examined chromatographically as above. These fresh onions could be grouped into three broad classes: (1) those with a high content of di-*n*-propyl disulfide and 5 to 9% of both methyl-*n*-propyl and *n*-propyl allyl disulfide; (2) those with a low amount of di-*n*-propyl disulfide and 10 to 16% methyl-*n*-propyl and *n*-propyl allyl disulfides; and (3) those with an intermediate amount of di-*n*-propyl disulfide and a concentration of methyl-*n*-propyl disulfide equal to one-half that of the *n*-propyl allyl disulfide, the latter two in the range of 5 to 10%. Correlation for this small group was 1.000.

When this knowledge was applied to the samples presented in Table 1, it was determined that each of the lot numbers could be classified as strong, medium, or mild according to this scheme. Thus for example lot number 501 would be ranked as a medium strength onion. Lot number 2327 would rank as a typical strong onion, and lot number 2595 would rank as a weak onion. Such an approach brings some order to Table 1.

It will be noted that soluble solids are also reported in Table 1. The author was unable to find any correlation between soluble solids content of fresh onions and any of the information obtained on the disulfides both as to kind and amount.

Examination of the genetic lines of all onion lots in this study likewise revealed no correlation between similar lines and disulfide distribution.

Total solids as determined by the vacuum oven method revealed no correlation with disulfide content.

The pungency ranking system outlined above was applied to the dried samples, but no obvious correlation was forthcoming. It is apparent that the relatively large differences between the various disulfide concentrations noted in the fresh samples (that is, the three group classification system) are not as well defined in the dried samples.

It was most difficult to obtain unequivocal subjective rankings of onion pungency. Even the word pungency has different meanings to various persons. To some, pungency is the eye irritating effect of the lachrymator; to others it is the chemotatic response of tongue burning or mouth irritation. Perhaps it is a combination of both effects or much more. Most assuredly the method outlined above needs additional refinement. We have initiated further investigations on this aspect of the program.

Table 2 allows one to estimate the concentrations of each of the disulfides within each lot and to compare the relative amounts of disulfides lost during dehydration (loss may also be interpreted as failure to produce the disulfides due to heat inactivation of the enzyme responsible for their production). Table 2 also permits comparison of concentrations between different lots.

Using the pungency ranking system described above, the author compared the total concentration of disulfides of each lot of fresh onions with its pungency ranking. The median value of total disulfide concentration for strong onions was 54,910 mm^2, moderate onions 33,820 mm^2, and mild onions 27,910 mm^2. This appears to indicate a correlation between the total concentration of the disulfides of fresh onion and this form of pungency ranking.

Median values for pungent, moderate, and mild dehydrated onions were as follows: 2,110 mm^2, 1,821 mm^2, and 2,037 mm^2. A suitable relationship appears to be marred by lack of meaningful subjective standards.

The quantity of disulfides lost in the transition from fresh tissue to dehydrated tissue may be estimated in several different fashions. (It should be noted that the data presented in Table 2 have not been corrected for sample size differences. Recall that 10 g fresh wt of onious were used to obtain the data listed under the headings "fresh," while 9 g of dehydrated onions plus 5 ml of water were used to obtain the data listed under "dried.")

Comparison can be made on a sample weight to weight basis, a total solids basis, on a drying ratio basis, or by a host of other means. The author found little agreement among various factions of the food processing industry as to just how this comparison should be made. Each individual contacted seemed to prefer his own or some facets of several methods.

The author finally chose to use the drying ratio method for comparison since there was least controversy concerning its use.

What is an appropriate drying ratio? Here again there was considerable difference of opinion. The author finally settled upon a compromise of 8:1, i.e., 8 g of fresh tissue yields 1 g of dehydrated tissue. This figure is generally accepted in the onion industry as being a reasonable one. Thus a 10-g fresh weight sample would be equivalent to a 1.25-g sample of dried material. Since 9 g of dried material were examined in this study, areas reported for a 9-g dried sample should be divided by a factor of 7.2. Since the reader may not find this means of comparison acceptable or useful for his purposes, data are presented in Table 2 simply as obtained under the experimental conditions noted.

Using an 8:1 drying ratio and the factor of 7.2, one can calculate the loss of volatile disulfides for samples quite readily. For example lot number 501 shows an 83% loss of dimethyl disulfide, 98% loss of methyl-*n*-propyl disulfide, 98% loss of methyl allyl disulfide, 99.9% loss of di-*n*-propyl disulfide, and a 99.4% loss of *n*-propyl allyl disulfide. There was a gain of 0.22% for diallyl disulfide. This gain seems to be atypical as compared to the other samples. Samples from lot number 3008 showed an 89% loss of dimethyl, a 98% loss of methyl-*n*-propyl, a 97% loss of methyl allyl, a 99.8% loss of di-*n*-propyl, and approximately a 100% loss of *n*-propyl allyl disulfides. Here

there was no detectable change in the amount of diallyl disulfide produced on dehydration.

Examination of all samples showed that total disulfide loss on dehydration ranged as high as 99.70% (lot number 3003) with all losses greater than 89%.

Measured loss of all volatiles (including disulfides) was estimated to average about 98% also.

It is interesting to speculate as to why one notes an increase in diallyl disulfide in some dehydrated onion samples. This could arise through ionic displacement by mercaptide ion:

$$RS^- + R'SSR' \rightleftharpoons RSSR' + R'S^-$$
$$\Updownarrow\ RS^-$$
$$RSSR + R'S^-$$

Fava *et al.* (1957) have shown the importance of this displacement even under mild conditions. Methyl, *n*-propyl, and allyl mercaptans have been reported in various *Allium* species (Wahlroos *et al.*, 1965).

This production could also reflect the increased thermal stability of the compound thus indicating that diallyl disulfide is more stable than many of the other disulfides in onions. The precise reason remains unknown.

The molar concentration of the volatile disulfides were calculated and they range from 10^{-8} *M* for the di-*n*-propyl disulfide in fresh onion samples to about 10^{-12} *M* for diallyl disulfide in the dried samples. Diallyl disulfide can quite possibly be present at levels below 10^{-12} *M*, but the sensitivity of the flame ionization detector is insufficient at these levels of concentration to detect it.

The most surprising facts disclosed by this study seem, first, to be the great loss of total volatile compounds and especially the volatile disulfides on dehydration and second, the rather complete realignment of the relative composition of all volatiles and again especially the disulfides on dehydration.

On first consideration, these figures may seem excessively large, but one must bear in mind that they reflect loss in volatile compounds only. Even with such losses, there is still a sufficient concentration of volatile components remaining in the dehydrated product to be readily detected by the human nose.

There is no real conflict between Copeman's findings and the author's. The quantities lost as reported in this study and the apparent lack of loss as reported by Copeman *et al.* (1947) is a matter that is easily resolved. The losses reported herein are only large in a relative sense. In absolute terms their total amount is undoubtedly far less than could be analyzed accurately by procedures available to researchers in 1947.

It now seems reasonable to assert that the major loss of volatiles takes place during the slicing or chopping operation just prior to drying. Indeed, this is reinforced by the observations of Haagen-Smit (Stephenson, 1949).

This analysis does not pretend to estimate the quantities of non-volatile flavor materials remaining in the sample. It is extremely difficult to estimate just how much of these materials is originally present in fresh onion tissue and equally difficult to estimate how much remains after completion of drying. There is no question that some flavor is lost and some changes occur, but one cannot deny that dehydrated onions do have excellent flavor capacity.

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C. BUZIASSY[a] and W. W. NAWAR
Department of Food Science, University of Massachusetts, Amherst, Massachusetts 01002

Specificity in Thermal Hydrolysis of Triglycerides

SUMMARY—Samples of triglycerides and triglyceride mixtures were heated in the presence of water under controlled conditions and the released fatty acids quantitatively analyzed by gas chromatography. Experiments with both a mixture of monoacyl-triglycerides and glycerides with equimolar amounts of randomly distributed fatty acids showed a preference for the hydrolysis of the shorter chain and the unsaturated fatty acids. The C_4, C_8, C_{12}, C_{16} and $C_{18:1}$ fatty acids were used in the above mixtures. A trilaurin, in which the fatty acid in the 2-position is labelled with C^{14}, was synthesized. When the free acids released by heat were analyzed by a combination gas chromatographic-radioactivity detector system, no evidence for a positional specificity was apparent.

INTRODUCTION

WHEN FATS ARE SUBJECTED to heat in the presence of moisture, hydrolysis of the ester bonds occurs, resulting in the liberation of free fatty acids. Several years ago, Lascary (1945, 1949) established that the reaction is essentially homogeneous and takes place within the oil phase rather than in the water-glyceride interface. Mills *et al.* (1949) concluded that the hydrolysis proceeds in a stepwise manner through diglycerides to monoglycerides to glycerol. Very little evidence, however, has been presented as to whether thermal hydrolysis involved any specificity with regard to the nature of the acid (fatty acid specificity) or to its location on the glyceride molecule (positional specificity).

Sahasrabudhe *et al.* (1964) heated corn oil for 2 days at 200°C in an open air oven, without addition of water, and concluded that there is a slightly higher susceptibility to deacylation in the primary positions. On the other hand when Crossley and co-workers (1962) heated pure 2-oleodipalmitin they observed the formation of free oleic and palmitic acids in the same ratio as that in the unheated glyceride (i.e., 1:2). Endres *et al.* (1962), in a study on thermal oxidation of synthetic triglycerides, reported that the hydrolysis occurred irrespective of the type and position of the fatty acid. Kintner *et al.* (1965) reported that the free fatty acid composition of milk, regardless of treatment, is comparable to the fatty acid composition of milk glycerides.

More recently, a communication from our laboratory reported the results of a study in which samples of corn oil, cottonseed oil and lard were heated at 200°C in the presence of moisture (Noble *et al.*, 1967). Although a preference for the hydrolysis of the shorter chain and unsaturated acids was observed, it was noted that data obtained from natural fats represent overall net effects in which more than one factor may be involved.

The object of the present study was to investigate specificity in thermal hydrolysis using simple model systems containing specific fatty acids in specific glyceride positions.

[a] Present address: Kellogg Co., Battle Creek, Michigan.

EXPERIMENTAL

Materials

Caprylic acid, lauroyl chloride, palmitoyl chloride, tricaprylin, trilaurin, tripalmitin (all 99% pure) and oleoyl chloride and triolein (95% pure) were purchased from Hormel Institute, Austin, Minnesota; trilaurin (99%) was purchased from Lachat Chemicals, Chicago Heights, Illinois; butyroyl chloride and tributyrin (reagent grade) from Fisher Scientific and lauroyl chloride 1-^{14}C from Nuclear Research Chemicals, Orlando, Florida. Capryloyl chloride and labelled trilaurin were synthesized in the laboratory. All triglycerides were further purified before use by repeated crystallization. In some cases gentle swirling of triglyceride solutions in ether with 5% $NaHCO_3$ was necessary to reduce the initial free fatty acid content. After purification, the triglycerides contained less than 1% free fatty acids and no other impurities, as determined by gas chromatography. In addition, no mono- or diglycerides could be detected by thin layer chromatography.

Gas-liquid chromatography (GLC)

The methyl esters were analyzed on an F&M Model 609 flame ionization gas chromatograph equipped with a 15-ft ⅛-inch diethelyne glycol succinate column. Peak areas were measured by means of a planimeter. When the system was tested with the quantitative methyl ester standard mixtures H-103 and K-108, supplied by Applied Science Laboratories, State College, Pennsylvania, the results agreed with the stated composition data with a relative error less than 5%. Reference mixtures were used frequently in order to insure adequate qualitative and quantitative performance of the column. Throughout this study, quantitative analysis was accomplished by the addition of appropriate internal standards to the samples before methylation and the use of correction factors for individual acids, as outlined in detail by Bills *et al.* (1963).

Analysis of fatty acids

To determine the total fatty acid composition (TFA), the glycerides containing the internal standards were refluxed with dry 10% HCl-methanol and the methyl esters recovered in petroleum ether as described by Stoffel *et al.* (1959). Factors relating the peak areas of individual esters to that of the internal standard were established from the analysis of a quantitative mixture of pure monoacyl-triglycerides. The total fatty acid content (i.e., free and esterified) of palmitic acid in a triglyceride sample, for example, was calculated as follows:

$$\text{Wt of total } C_{10} \text{ in triglyceride} = \frac{\left[\text{Peak area of } C_{10} \text{ ester}\right] \times \left[\text{factor for } C_{10}\right] \times \left[\text{Wt of internal standard}\right]}{\text{Peak area of internal standard}}$$

Heptadecanoic and tridecanoic acids were used as the internal standards.

The free fatty acid (FFA) analysis was carried out according to the method described by Hornstein *et al.* (1960), in which the free acids are adsorbed and methylated on a basic resin. Factors for each acid were similarly established from the analysis of a quantitative mixture of pure free fatty acids. Thus, two correction factors were used for each fatty acid; one for determination of the total acid present, by the method of Stoffel, and the other for analysis of the free acid, by the method of Hornstein.

To minimize the loss of short-chain fatty acid methyl esters during their recovery from the interesterification medium, the procedure of Bills *et al.* (1963), in which ethyl chloride is used in place of petroleum ether and the solvent evaporation is carried out with partial refluxing, was followed. However, some modifications were made to raise the efficiency of the method and allow for simultaneous processing of eight samples.

Simultaneous "gas chromatographic-radioactive counting" analysis

A combination system was used in which an F&M Model 810 flame ionization gas chromatograph was attached to a Nuclear Chicago "Biospan" Model 4998 gas radiochromatography counter. An internal 4 pi gas flow proportional detector measured radioactivity directly in the chromatograph effluent. The capacity of the detector chamber was 86 ml. The combination system was equipped with a two channel strip chart recorder.

Thermal hydrolysis

The triglyceride samples, each approximately 100 mg, were placed in 2-ml vials and the appropriate amount of CO_2-free water added. The tubes were then closed under a nitrogen atmosphere with tight screw caps lined with silicone rubber septa and heated in an open air oven. After heat treatment, the vials were removed from the oven, cooled at 5°C and their contents analyzed. The percentage hydrolysis of the individual fatty acids was calculated from the formula (using palmitic acid as an example):

$$\%\ \text{hydrolysis of } C_{16} \text{ acid} = \frac{\left[\text{Wt of free } C_{16} \text{ after heating}\right] - \left[\text{Wt of free } C_{16} \text{ before heating}\right]}{\left[\text{Wt of total } C_{16} \text{ in triglyceride}\right] - \left[\text{Wt of free } C_{16} \text{ before heating}\right]} \times 100$$

RESULTS AND DISCUSSION

THE FOLLOWING CONDITIONS were selected as the parameters of hydrolysis: a water concentration of 100% (w/w), a temperature of 200°C and a heating period of 2.5 hr unless otherwise specified. These conditions resulted in an appropriate level of hydrolysis (10–30%); a level not too low for repeatable and accurate measurement and not high enough to cause loss of specificity. In addition, it was found that under these conditions no randomization or reesterification could be detected (Noble *et al.*, 1967).

Fatty acid specificity in thermal hydrolysis

In order to study fatty acid specificity, any possibility of positional preference in thermal hydrolysis must be eliminated. For this purpose two approaches were followed. First the hydrolysis of equimolar mixtures of monoacyl-triglycerides was studied. In the second approach, triglycerides with equimolar amounts of randomly distributed fatty acids were employed. In both experiments the fatty acids selected were butyric, caprylic, lauric, palmitic and oleic acids.

The wide range of volatility and gas chromatographic retention time of these fatty acids made it desirable to divide their triglycerides into two groups, each prepared, heated and analyzed separately. One group contained the glycerides of butyric, caprylic and lauric acids; the other contained lauric, palmitic and oleic acids. Lauric acid served as a link to correlate the results of the two groups.

Hydrolysis of equimolar mixtures of monoacyl-triglycerides

Equimolar amounts of the triglycerides were mixed, melted and several aliquots assayed for FFA and TFA. One hundred mg portions (12 aliquots) were then transferred into vials, the appropriate amount of water added and the samples heated. The heat-hydrolyzed fatty acids were quantitatively analyzed. This experiment was repeated 12 times and the significance of difference in "percent hydrolysis" was determined by the student "t" test for paired data.

From the results summarized in Table 1, a progressive preference of hydrolysis in favor of the shorter chain fatty acids is apparent. If this pattern is extrapolated to the C_{18} saturated acid, it is expected that its hydrolysis rate would be lower than that of the 16-carbon fatty acid. However, the hydrolysis rate of oleic acid falls between that of the saturated acids with 12 and 16 carbons. Unsaturation appears to raise the rate of hydrolysis.

Hydrolysis of triglycerides with randomly distributed fatty acids

The glycerides were synthesized according to a modification of the procedure described by Hartman (1957). An equimolar mixture of fatty acid chlorides sufficient to esterify one primary position on the glycerol present was reacted. The resulting product, after standing overnight,

Table 1. Hydrolysis of equimolar mixtures of monoacyl-triglycerides heated at 20°C for 2.5 hr.

Acid	No. of trials	% Hydrolysis[1]		Standard Error of the Mean
Triglyceride mixture A				
4:0	11	46.9	**	2.35
8:0	11	29.6		0.95
12.0	11	27.5	**	0.95
Triglyceride mixture B				
12:0	12	20.5	**	0.93
16:0	12	17.8		1.12
18:1	12	18.0	N.S.	0.85

[1] Calculated according to equation shown in text.
** Difference between the two means included in bracket, significant beyond the 1% level.
N.S. Not significant.

Table 2. Thermal hydrolysis of triglycerides with equimolar quantities of fatty acids randomly distributed.

Acid	No. of trials	% Hydrolysis[1]		Standard Error of the Mean
Triglycerides A				
4:0	12	54.2	**	1.36
8:0	12	31.6		0.58
12:0	12	27.2	**	0.84
Triglycerides B				
12:0	12	18.0	**	0.50
16:0	12	12.0		0.24
18:1	12	16.6	**	0.31

[1] Calculated according to equation shown in text.
** Difference between the two means included in bracket, significant beyond the 1% level.

showed no presence of diglycerides. The monoglycerides were then esterified with a second equimolar mixture of fatty acid chlorides to form the 1,3 diglycerides, which were also allowed to stand overnight. The final step was the esterification of the secondary position with a slight excess of the equimolar fatty acid chloride mixture. Total fatty acid and free acid contents of the synthesized triglycerides were then determined and the random distribution of the fatty acids was confirmed by lipase hydrolysis. Enzymatic digestion was carried out essentially as described by Tattrie and co-workers (1958).

Twelve aliquots of the synthesized product were then heated and analyzed as described above. The results are shown in Table 2. The progressive preference for the hydrolysis of the shorter chain fatty acids is again apparent. The hydrolysis rate of the unsaturated 18-carbon fatty acid also fell between those of the 12- and 16-carbon saturated acids, showing a possible preferential hydrolysis of the unsaturated acid.

The fatty acid specificity observed in this study is probably due to the greater accessibility of water to the ester bond in case of the shorter and unsaturated acids. The results presented here on triglycerides are in agreement with the earlier observations of Noble *et al.* (1967) on natural fats.

Data in Tables 1 and 2 show that the C_{12} acid was hydrolyzed to a greater extent when heated in the presence of shorter chains than when in the presence of longer chain acids. This is probably due to the more hydrophilic nature of glycerides containing shorter chain acids and the consequently greater solubility of water in the glyceride mixture. In addition, since shorter chain acids are liberated more rapidly, the free fatty acid content of the system will be higher and this in turn increases the rate of the hydrolysis reaction (Lascaray, 1952).

Positional specificity in thermal hydrolysis

To determine whether the position of the fatty acid on the glyceride molecule has an effect on the rate of its thermal hydrolysis, a monoacyl-triglyceride, trilaurin, was synthesized with the fatty acid on the 2-position labelled with C^{14}. First the 1,3-dilaurin was prepared, repeatedly crystallized and then reacted with labelled lauroyl chloride. The final product was recovered with a yield of 60% and did not show any detectable contamination of free acid, mono- or diglycerides when examined by TLC. When an aliquot of the labelled glyceride was subjected to approximately 10% hydrolysis by lipase, only a trace of activity could be detected in the free acids confirming that the label was essentially in the 2-position.

Table 3. Thermal hydrolysis of C^{14}-labelled trilaurin.

	No. of Samples	Mean
Percent hydrolysis of C_{12} acid	14	9.53 ± 0.78
Rel. activity of TFA before heating	8	2.38 ± 0.12
Rel. activity of thermally hydrolyzed FFA	14	2.34 ± 0.13

The fatty acids and their activity were measured by means of a combination "gas chromatograph-flow counter," which provides simultaneous measurement of fatty acids and their radioactivity. A value representing the "relative activity" of the fatty acid was obtained by dividing the area of the radioactivity peak by that of its corresponding GLC peak.

Fourteen aliquots of the labelled trilaurin were separately heated and analyzed. As shown in Table 3, there was no significant difference between the relative activity values of the acids released by heat and those of the total fatty acids before heating. Under the experimental conditions used here, no positional specificity could be detected.

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OLOF SAMUELSON and HANS STRÖMBERG
Department of Engineering Chemistry, Chalmers Tekniska Högskola, Göteborg, Sweden

Alditol Determination in the Presence of Saccharides

SUMMARY—Alditols and sugars were extracted with water from sweets, and after dilution with ethanol the solutions were subjected to partition chromatography on ion-exchange resins. The alditols and monosaccharides were determined automatically using the periodate and orcinol methods.

INTRODUCTION

PARTITION CHROMATOGRAPHY ON ION EXCHANGE RESINS in aqueous ethanol is a useful method for the analysis of complicated mixtures of sugars (Arwidi *et al.*, 1965; Larsson *et al.*, 1965). Recently, sugar alcohols have been separated by the same technique and determined both in the presence and absence of sugars (Samuelson *et al.*, 1966). no applications of this technique for practical analyses have been studied previously.

Sugar alcohols, mainly glucitol (sorbitol), are added to various dietetic foods during their manufacture, and a number of methods for their determination have been published (Genset *et al.*, 1962, Adcock, 1957).

This paper describes a chromatographic method suitable for the determination of glucitol, mannitol and other alditols in foods. Several sugars can be determined in the same run.

EXPERIMENTAL

AS IN PREVIOUS WORK, most of the separations were made on a strongly basic anion exchanger in the sulfate form [Technicon T5 C; 7 to 11 μ (Larsson *et al.*, 1966)]. As a check of identity of the alditols, runs were also made with a cation exchange resin in its potassium form [Dowex 50 W X-8, 14–17 μ].

The chromatographic separations were made with the same type of equipment as described previously, and the working conditions were similar to those employed in earlier work on alditol separations. Experimental details are given in the legends under the chromatograms.

The analyses of the eluates were made automatically by connecting the outlet of the ion exchange column directly to a monitor for colorimetric analysis. Both sugar alcohols and sugars were determined by the periodate method (Samuelson *et al.*, 1966). The sugars were also determined in a separate channel using the orcinol method (Arwidi *et al.*, 1965), which gives no response with sugar alcohols. The analyses were made in a Technicon Auto-Analyzer.

The samples investigated were a dietetic chocolate and a toffee, both of commercial origin. A laboratory-made toffee also was analyzed. The water extracts obtained from the samples were centrifuged and absolute ethanol added to obtain an ethanol concentration (w/w) of 84%. The samples were then allowed to stand in a refrigerator for 24 hr to complete the precipitation of insoluble material. An aliquot of the clear supernatant solution was withdrawn with a micropipet and a sample containing about 250 μg of the main alditol (glucitol) was applied to the chromatographic column.

RESULTS AND DISCUSSION

THE CHROMATOGRAM RECORDED with the periodate method in a run with the extract from a dietetic chocolate on an anion exchanger in its sulfate form is given in Fig. 1. The main chromatographic band denoted by G-1 had a peak elution volume corresponding to that of glucitol. The minor band (M-1) had a position in good agreement with that obtained with an authentic sample of mannitol. A trace amount of some unknown solute was recorded immediately after the main band. Very small amounts of unknown solutes were recorded at low peak elution volumes. The peak positions indicated that these peaks contained solutes with relatively few hydroxyl groups.

No monosaccharides or disaccharides were recorded in the second channel of the monitor in which the analysis was made by the orcinol method. This means that the total concentration of monosaccharides and disaccharides was less than 0.5%.

The identities of glucitol and mannitol were confirmed by a run on a cation exchange resin in its potassium form. With this resin the order of elution of these alditols is reversed.

Chromatograms from runs on the anion exchanger were used for quantitative determinations of the alditols. The areas under the elution curves were determined as usual (Moore *et al.*, 1958) and compared with those obtained in calibration runs with known amounts of authentic substances. The sample was found to contain 38.7% glucitol

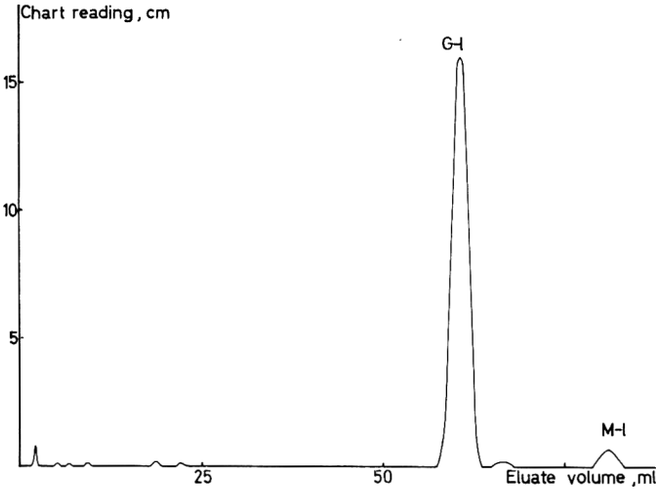

Fig. 1. Chromatogram of chocolate. Loading: 0.82 mg. Resin: T5C, sulfate form, 7–11 μ. Resin bed: 2.6 × 875 mm. Flow rate: 2.8 ml cm^{-2} min^{-1}. Temperature: 75°C. Eluant: 86% ethanol w/w. Analysis: neutral periodate.

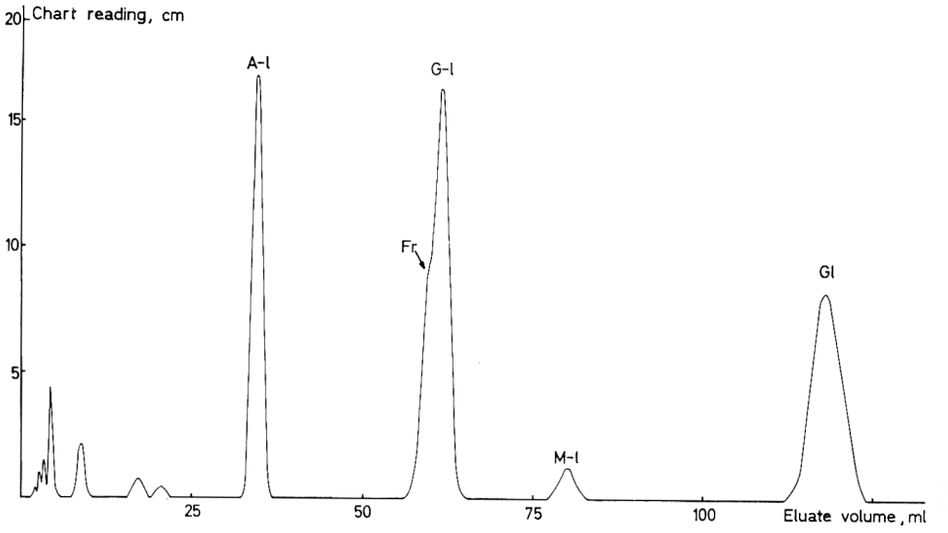

Fig. 2. Chromatogram of toffee with arabinitol (0.12 mg) as internal standard. Loading: 5.22 mg. Other variables as in Fig. 1.

and 0.8% mannitol. In repeated runs the deviations in the glucitol determinations were ± 0.8% from the mean or less. The maximum deviation in the mannitol determinations was less than 0.02%. In the runs used for the mannitol determinations, larger samples were added to the chromatographic column allowing the glucitol peak to run off scale. The values found agreed well with the analysis given by the manufacturer (39.2% hexahydric alcohol).

The toffee which, according to the manufacturer, contained glucitol and starch sirup as main sweetening agents gave a more complicated pattern. The chromatogram recorded in the periodate channel from a run on an anion exchange column is given in Fig. 2.

Arabinitol (A-l) was added as an internal standard in this run. In agreement with the data given by the manufacturer, a significant band with the position of glucitol was recorded (denoted by G-l). The front exhibited a shoulder indicating the presence of some overlapping compound with a peak elution volume slightly lower than that of glucitol. Fructose (Fr) is known to appear at this position as was confirmed by model experiments on the same column. By increasing the column length to 1,130 mm and the ethanol concentration to 96%, two peaks were obtained but the curves still overlapped slightly. Another main peak with the position of glucose (Gl) was recorded as well as a minor peak the position of which agreed with that of mannitol (M-l). The minor compounds which were recorded at very low peak elution volumes had positions typical for solutes containing a lower number of hydroxyl groups. No attempt was made to identify these.

Disaccharides and higher saccharides were eluted after glucose but these bands were not investigated further. In the orcinol channel the peaks with positions corresponding to glucose, fructose and higher saccharides were recorded. As expected, no peaks were recorded at the positions corresponding to those of glucitol and mannitol, which give no color reaction with this reagent.

In a separate run the periodate oxidation was made at pH 1 instead of pH 7.5. Under these conditions, glucose and other aldohexoses give only a weak response, whereas alditols give approximately the same response at both pH-values. Periodate oxidation at low pH is used to advantage in analysis of solutions containing aldoses with peak positions very close to those of the alditols present in the sample. Unfortunately, fructose gives a strong response also in this medium and the choice of a low pH does not facilitate the quantitative determination of glucitol in the presence of fructose. On the other hand, chromatograms recorded under these conditions are useful for identification purposes. The chromatogram of the water extract from the toffee confirmed the presence of glucitol, mannitol, fructose and glucose. A final confirmation was obtained in a chromatographic run on a cation exchanger in the potassium form.

In the quantitative analysis of this sample, glucose and fructose were determined as usual from the chromatogram recorded in the orcinol channel in runs on the anion exchanger. Mannitol, which is well separated from other solutes on the anion exchanger, was determined from the elution curve recorded in the periodate channel. The area corresponding to the sum of sorbitol and fructose was determined from the same chromatogram and compared with calibration runs with solutions containing the same amount of fructose as determined in the orcinol channel plus varying amounts of glucitol.

The analysis gave the following results: glucitol 4.1 ± 0.1%; mannitol 0.2 ± 0.01%; fructose 1.6 ± 0.1%; glucose 7.2 ± 0.2%. The hexitol determinations were in agreement with the value given by the manufacturer (4.3% hexahydric alcohol). The sugar composition was not known and since the accuracy of the ion exchange method has been well established in previous work no determinations with other methods were made.

The laboratory-made toffee was prepared by mixing 20 g of a technical glucitol solution containing 58.9% glucitol and 3.1% mannitol with 180 g "toffee-base" (free from sugar alcohol). The sample was analyzed both by the anion-exchange and the cation-exchange methods. A chromatogram from a run on a cation exchange column is reproduced in Fig. 3. The bands corresponding to

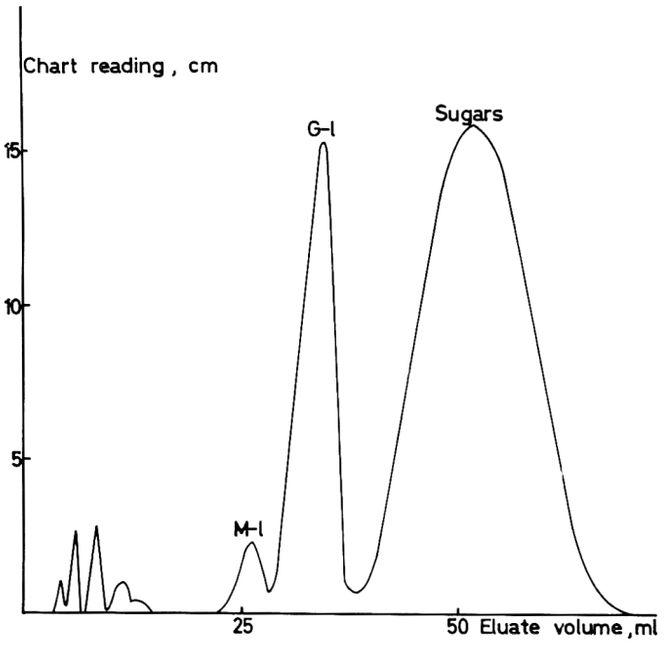

Fig. 3. Chromatogram of synthetic toffee. Loading: 3.12 mg. Resin: Dowex 50 W-X 8, potassium form, 14–17 μ. Resin bed: 2.6 × 1310 mm. Flow rate: 5.6 ml cm^{-2} min^{-1}. Temperature: 75°C. Eluant: 80% ethanol w/w. Analysis: neutral periodate.

mannitol (M-l) and glucitol (G-l) had the same positions as in runs with authentic samples and appeared before the sugars, which were not separated from each other under these working conditions. The fast moving compounds which appeared before mannitol were recorded with the technical glucitol solution as well and with the exception of the sugar band the chromatogram was identical with that obtained with this solution.

The results of the quantitative determinations of mannitol, glucitol and glucose are given in Table 1. The sugar alcohols were determined by the periodate, and glucose by the orcinol, method. Glucose was only determined in the run on the anion exchanger since serious overlapping with disaccharides occurred on the chromatogram from the cation exchanger. As shown in the run on the anion exchange column, fructose was not present in the sample. It is seen that good agreement was obtained between the amounts added and those found analytically.

Table 1. Analysis of synthetic toffee.

	Added, %	Found, %	
		Anion exchange	Cation exchange
Mannitol	0.31	0.30	0.30
Glucitol	5.9	6.0	6.1
Glucose	4.9	4.9	.....

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R. W. PORTER*
Fisheries Technological Laboratory
U. S. Bureau of Commercial Fisheries, Ketchikan, Alaska

The Acid-Soluble Nucleotides in King Crab Muscle

SUMMARY—Nucleotides in rested king crab muscle were identified as NAD, AMP, GMP, IMP, ADP, UDPAG, UDPG, ATP, GTP and UTP. Of three additional minor nucleotide fractions, two are tentatively thought to contain NAD; the third remains unidentified. The nucleotide profiles of three different leg sections were very similar, with ATP the predominant nucleotide. Average total nucleotide content of these three sections was 3.47 $\mu M/g$. Muscle from severely exhausted crabs held frozen overnight contained 0.35 $\mu M/g$ of IMP. Since king crabs are processed fresh and IMP does not accumulate to a very high level, IMP probably does not play an important role in king crab flavor.

INTRODUCTION

THE WELL ESTABLISHED role of nucleotides as flavor enhancers has been reviewed recently (Kuninaka *et al.*, 1964; Kuninaka, 1966). Considerable data exist on nucleotides in fish (Jones *et al.*, 1960; Tarr *et al.*, 1962; Kobayashi, 1966), in shrimp (Tarr *et al.*, 1965), and in several other species of marine invertebrates (Nakajima *et al.*, 1961, Arai, 1966).

The rate of nucleotide degradation, particularly IMP, in various species of fish held in ice or in frozen storage has been the subject of several investigations. The importance of IMP in fish flavor is indicated by the use of its degradation to hypoxanthine as an index of the loss of quality in fish stored in ice (Jones *et al.*, 1964; Spinelli *et al.*, 1964).

Terasaki *et al.* (1965) studied the nucleotides in meat from chickens, pigs, sheep, and horses and found that maximum IMP content varied with species and method of slaughter. Dannert *et al.* (1967) reported a variation in IMP content among species of meat animals but no difference in IMP content among pork muscles. Except for the muscle along the lateral line, Spinelli (1967) found no difference in IMP and hypoxanthine content from different locations within the halibut.

Arai (1966) followed the degradation of AMP, IMP, adenosine, and inosine in crude extracts of muscle from various marine invertebrates. He concluded the major pathway of ATP degradation in marine invertebrates proceeds as follows: ATP→ ADP→ AMP→ adenosine→ inosine→ hypoxanthine. He also noted a small degree of adenylic acid deaminase activity in the crustaceans but not in the other invertebrates.

Unlike red meat and fish which normally have a storage period while raw, king crabs are cooked within 15 to 20 min after they were butchered. Therefore, the buildup and subsequent breakdown of IMP normally observed in red meat and fish during post-mortem storage would not appear to have an opportunity to proceed in king crab.

The objectives of this study were: (1) to identify the nucleotides in rested king crab muscle; (2) to determine the nucleotide profile on different sections of the crab; and (3) to measure the extent of IMP accumulation in the muscles of exhausted crab.

MATERIALS AND METHODS

Samples and extraction

King crabs are kept alive in tanks supplied with fresh sea water before processing. For experiments at Ketchikan, Alaska, male king crabs (6 to 8 lb.) were shipped alive by air from Seldovia, Alaska, and placed in a live-holding tank. The crabs were allowed to rest for at least 24 hr before use.

Samples for nucleotide identification were obtained by killing the crab and immediately removing the raw meat from the shell and placing it in a beaker held in an ice bath. The tissue was ground once through 1/8-in. plate in a pre-chilled grinder and quickly mixed to give a uniform sample. Fifty-gram samples were immersed in 2 volumes of chilled 0.6 *N* perchloric acid and homogenized for 2 min.

After filtration under suction, the pulp was washed twice with a small volume of 0.6 *N* perchloric acid. All of these operations were done in a cold room (0 to 4°C). The filtrate was adjusted to pH 6.5 to 6.8 with 10% KOH and allowed to stand at −10°C until ice-crystals started to appear. The insoluble potassium chlorate was removed by filtration and the clear solutions were stored at −50°C until analyzed.

Two crabs were used for measuring the nucleotide profile in different sections of the animal. After they were allowed to rest for 24 hours in the holding tank, the crabs were butchered, and the meat removed from the shell. The meat from the following sections of the crab was combined for analysis: (1) both claws, (2) merus section of all 6 walking legs, and (3) propus section of all 6 walking legs.

Nucleotide extracts were prepared by placing samples from the three sections in separate blenders and simultaneously immersing in perchloric acid. Simultaneous treatment prevented any difference due to enzymatic changes, which could occur if the sections were extracted sequentially.

Six male king crabs (6 to 8 lb) were used to measure the IMP accumulating in the muscles of exhausted crab. They were subjected to the usual commercial handling before they were butchered. They were taken from the holding tanks and piled on their backs in a cart for transfer to the butchering station.

About 1 hr elapsed between removal from the tank and butchering. During this time the crabs continually struggled to right themselves. At this point, care was taken to sample exhausted crabs that were still alive. They were

*Present address: Central Research Department, Anheuser-Busch, Inc., St. Louis, Mo.

quickly butchered and allowed to drain free of blood for 10 min. The meat was then frozen in the shell and held overnight at −29°C. After removal from the shell the meat was extracted with perchloric acid and the acid extracts frozen at −37°C. They were packed in dry ice, and shipped to Ketchikan where they were held at −50°C for analysis.

Column chromatography

Samples representing 10 to 16 g of crab meat were placed on 1.0 × 28.0 cm columns of Dowex 1 × 8 (Formate) 200 to 400 mesh. The columns were washed with water until the column effluent was free of material absorbing at 260 mμ. The nucleotides were eluted according to the procedure of Jones *et al.* (1960) except that 0.5 *N* formic acid (600 ml) and 2.0 *N* formic acid (320 ml) were substituted, in that order, for the 2.0 *N* formic acid (470 ml) in the original procedure. The columns were run in a cold room at 0 to 4°C. The effluent from the columns was continuously monitored at 260 mμ with a Vanguard model 1056 A UV monitor. Fractions common to the individual peaks were pooled and then freeze-dried for further analysis.

Thin-layer chromatography

Polyethyleneimine-cellulose anion exchange thin-layer plates 0.5 mm thick were prepared according to Randerath (1963), using MN300 cellulose powder (Brinkmann Instrument Co., Westbury, New York), and Polymin p (50% aqueous solution of Polyethyleneimine, Chemirad Corp., East Brunswick, New Jersey). The plates were dried overnight at room temperature, covered, and stored in a refrigerator until used. Good resolution was obtained only after the plates had been stored for about 48 hr in the refrigerator.

Approximately 10^{-3} μmoles of known (Sigma Chemical Co., St. Louis, Missouri) and unknown nucleotides from minor UV absorbing peaks were spotted on the plates. After spotting, the plates were desalted in anhydrous methanol for 10 to 12 min (Randerath, 1964) and dried under a cool stream of air prior to development in LiCl. The concentration of LiCl varied with the nucleotides chromatographed: NAD and nucleoside monophosphate (0.20 *M*), uridine diphosphate sugars (0.25 *M*), nucleoside diphosphates (1.0 *M*), and nucleoside triphosphate (1.5 *M*). After development the plates were dipped in 0.003% fluorescein solution to increase the contrast and were dried prior to examination under shortwave UV light (Mineralight, Model R-51).

Spectrophotometric examination

The combined fractions from each peak were concentrated by freeze-drying and the spectra of each peak were determined in 0.01 *N* HCl and 0.002 *N* NaOH. The UV spectra, 250/260 and 280/260 mμ absorbing ratios were compared with literature values (Anon., 1956). The absorption peaks were quantitatively evaluated, using published molar extinction coefficients (Dorough *et al.*, 1954; Bock *et al.*, 1956).

Chemical analysis

Inorganic phosphate was determined by the method of Rockstein *et al.* (1951), after the samples were digested for labile or total phosphorus according to Hurlburt *et al.* (1954). Ribose was determined by the procedure described by Hurlburt *et al.* (1954), using AMP as a standard. Peaks suspected of containing hexoseamines were assayed using the p-dimethylaminobenzaldehyde reaction (Ashwell, 1957), and peaks suspected of containing sugars were checked by the anthrone test (Scott *et al.*, 1953).

RESULTS AND DISCUSSION

Nucleotide identification

A typical elution pattern of nucleotides from rested king crab muscle is presented in Figure 1. A summary of the identification data for the various peaks is presented in Table 1. Peaks 1, 2, and 3 were all found to contain NAD as indicated by thin-layer chromatography. The absorption spectra of Peak 1 showed a broad region of strong absorption between 250 and 275 mμ but did not show a distinct maximum or minimum absorption wavelength at either acid or alkaline pH.

Occasionally, a spot corresponding to CMP would appear when large amounts of material from Peak 1 were chromatographed on thin-layer plates. The absorption spectra of Peak 2 showed strong absorption between 245 and 260 mμ with no clearly defined maximum or minimum. Peak 5 was generally found in small quantity and was unidentified. Slightly larger amounts were observed in crabs that had been under stress.

Ribose and phosphate analyses were possible only on a few well-separated fractions that occurred in fair quantities. Sugar phosphates and inorganic phosphate were found to elute in the same region as IMP and interfered with the phosphate and ribose analyses on this peak.

The presence of N-acetyglucosamine in Peak 9 was confirmed by the para-dimethylaminobenzaldehyde test. This peak ran equivalent to known UDPAG on thin-layer chromatograms. Peak 10 gave a positive response to the anthrone test and ran equivalent to known UDPG on thin-layer chromatograms. Peaks 12 and 13 were identified as GTP and UTP respectively. Thin-layer chromatography showed that each was slightly contaminated by the other.

The presence of the mono- and di-phosphate compounds

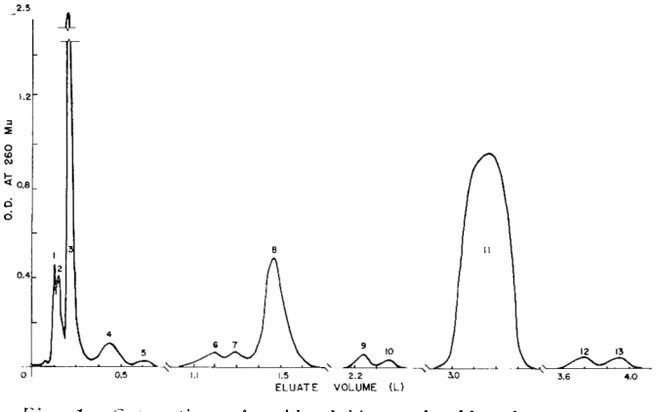

Fig. 1.—Separation of acid-soluble nucleotides from 16 g of king crab muscle on Dowex 1-X8 (Formate). Details of chromatographic conditions are in the text. 1 = NAD plus a contaminant; 2 = NAD plus a contaminant; 3 = NAD; 4 = AMP; 5 = unknown; 6 = GMP; 7= IMP; 8 =ADP; 9 = UDPAG; 10 = UDPG; 11 = ATP; 12 = GTP; 13 = UTP.

Table 1. Summary of data on nucleotide peaks separated by column chromatography.

Fraction	Nucleotide	pH 2.0 Max Mμ	pH 2.0 Min Mμ	pH 11.0 Max Mμ	pH 11.0 Min Mμ	Ratios @ pH 2.0 250/260	Ratios @ pH 2.0 280/260	TLC	Ribose M/M Base	PO 4 M/M Base Labile	PO 4 M/M Base Total
1	* NAD[1]	**	**	**	**	**	**	Yes	**	**	**
2	* NAD[1]	**	**	**	**	**	**	Yes	**	**	**
3	NAD	261	234	260	233	....	....	Yes	.64	0	1.65
4	AMP	257	231	259	228	.85	.30	Yes	.99	0	1.05
5	Unident.	.....	.....	.....	.....	....	....	No	....	....	....
6	GMP	257	230	258	232	.93	.60	Yes	.92	0	1.18
7	IMP	250	226	253	232	1.35	.34	Yes	**	**	**
8	ADP	257	230	259	230	.82	.30	Yes	.93	.70	1.88
9	UDPAG	262	234	260	238	.85	.46	Yes	**	**	**
10	UDPG	261	232	259	244	.86	.47	Yes	**	**	**
11	ATP	257	232	259	227	.84	.25	Yes	.89	2.10	3.10
12	GTP	255	230	258	231	.99	.73	Yes	**	**	**
13	UTP	261	233	261	242	.83	.42	Yes	**	**	**

* Tentative identification based on thin-layer chromatography.
** Unable to determine.
[1] = contaminant.

when the tri-phosphate compounds were developed on thin-layer chromatograms, suggests that breakdown of the tri-phosphates had occurred during concentration.

The nucleotides found in king crab muscle are similar to those observed in rested cod by Jones *et al.* (1960). In addition to those observed in king crab, they found very small amounts of other nucleotide constituents (TPN, GDP, CTP, UDP and UDPGA). In the present study, the di-phosphates of guanosine and uridine were not found as such, probably because they were eluted in indistinguishable amounts with larger fractions. The mono-phosphate of uridine was not found, and only small amounts of GMP were observed, probably for the same reason. The presence of all of these compounds is implied by the fact that GTP and UTP were found in rested muscle.

Nucleotide content in different crab sections

A summary of the nucleotide content of the merus, propus, and claw sections from rested king crab is presented in Table 2. In all three sections, ATP was the predominant nucleotide present. The merus and claw sections had nearly the same nucleotide content of 3.77 and 3.55 $\mu M/g$ respectively. The propus section contained somewhat less total nucleotide (3.10 $\mu M/g$). This smaller amount is probably due to the fact that this section is characteristically flatter than are the merus or the claw and contains a larger proportion of connective tissue and skin. Very little IMP was found in any of the three sections studied in rested crab. In each section, the IMP level was less than 0.10 $\mu M/g$. The total nucleotide in the three sections averaged 3.47 $\mu M/g$ and is considerably less than that reported for most other species.

Arai (1966) reported a total nucleotide content of 5.57 $\mu M/g$ in the leg muscle of another species *Paralithodes brevipes*. However, he also found IMP in the smallest concentration (0.16 $\mu M/g$), and ATP in the highest concentration (3.51 $\mu M/g$) among the nucleotides measured. The difference in total nucleotides found by Arai (1966) in *Paralithodes brevipes* and that found in *Paralithodes camtschatica* here could have been attributed to the molt stage of the crabs at the time of sampling as well as species differences.

Table 2. Nucleotide content of muscle tissue from leg sections of king crabs.

Nucleotide	Merus	Propus	Claws
		$\mu M/g$ wet weight tissue	
NAD	0.21	0.09	0.18
AMP	0.08	0.06	0.12
GMP	0.04	0.02	0.03
IMP	0.06	0.06	0.04
ADP	0.37	0.17	0.43
UDPAG	trace	0.04	0.07
UDPG	trace	0.03	0.05
ATP	3.01	2.63	2.63
GTP	trace	trace	trace
UTP	trace	trace	trace
Total	3.77	3.10	3.55

Nucleotides in exhausted crabs

An accumulation of IMP has been observed in exercised fish muscle (Jones *et al.*, 1960; Guardia *et al.*, 1965). Since it has been shown that maximum flavor is related to maximum IMP content in meat (Teraski *et al.*, 1965), we hypothesized that extreme fatigue might result in an accumulation of IMP and an improvement in the flavor of king crab. The nucleotide content of muscle from severely exhausted king crab is shown in Table 3.

Table 3. Nucleotide content of exhausted crab.

Nucleotide	$\mu M/g$ wet weight
NAD	.07
AMP	.91
GMP	.11
IMP	.35
ADP	.20
UDPAG	trace
UDPG	trace
ATP	.....
GTP	.....
UTP	.....
Total	1.64

The ATP was completely depleted as expected, and only a small amount of ADP was observed (0.20 $\mu M/g$). The largest single nucleotide pool was in the form of AMP (0.91 $\mu M/g$). These results indicate that IMP accumulates only to a limited degree during severe ante-mortem exhaustion of king crab (.35 $\mu M/g$). Although the post-mortem degradation of nucleotides in king crab was not included in this study, it is assumed to be unimportant due to the short time lapse between butchering and cooking under commercial conditions. The author feels that there is little likelihood that IMP plays a significant role in the flavor of king crab.

ABBREVIATIONS USED

NAD—nicotineamide adenine dinucleotide
AMP—adenosine 5′-monophosphate
ADP—adenosine 5′-diphosphate
ATP—adenosine 5′-triphosphate
GMP—guanosine 5′-monophosphate
GDP—guanosine 5′-diphosphate
GTP—guanosine 5′-triphosphate
UDP—uridine 5′-diphosphate
UDPG—uridine 5′-diphosphate glucose
UDPAG—uridine 5′-diphosphate N-acetylglucoseamine
UDPGA—uridine 5′-diphosphate glucuronic acid
UTP—uridine 5′-triphosphate
TPN—triphosphopyridine nucleotide
CMP—cytidine 5′-monophosphate
CTP—cytidine 5′-triphosphate
IMP—inosine 5′-monophosphate

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R. TOLEDO, M. P. STEINBERG and A. I. NELSON
Department of Food Science, University of Illinois, Urbana, Illinois

Quantitative Determination of Bound Water By NMR

SUMMARY—A Nuclear Magnetic Resonance (NMR) instrument was used to measure the amount of liquid water in a complex colloidal system over a broad temperature range. The bound water content, defined as that which remained liquid at 0°F (−18°C), was 0.29 ± 0.01 g water per g dry solid in case of a wheat flour dough. This value was independent of total moisture content for doughs of the same flour with moisture contents greater than 24.6%. NMR signals indicated that most of this water remained liquid at −58°F (−50°C). This method gives a direct reading of bound water and is non-destructive of sample.

INTRODUCTION

SCIENTISTS HAVE NOT AGREED upon a definition of "bound water" because there is no standard method for its determination (Kuprianoff, 1958). This term is generally taken to indicate water that is so closely united with other compounds in the system that its properties are different from the remainder or "free water." Little information is available on the relation of bound water to food quality because the estimation methods are not only difficult and time consuming but also of poor accuracy.

According to Meryman (1966), "Current usage in cryobiology loosely defines bound water as that which does not freeze." Mazur (1966) concluded that the unfrozen water in cells is water bound to cellular solids. Kuprianoff (1958) concluded that the most reliable method to date for determination of bound water is to measure the water that persists in the liquid phase at low temperatures. This can be done by calorimetric or dilatometric methods indirectly, i.e., the heat lost by a product in cooling to 0°F (−18°C) indicates the amount of ice present so that the unfrozen or bound water must be obtained by difference from the total water.

There is little doubt that biochemical systems contain liquid water at sub-freezing temperatures. Pichel (1965) found that a considerable portion of the water in egg whites remains liquid at low temperatures and the amount of this bound water decreases with denaturation. Mannheim *et al.* (1957) using a calorimetric method found that water in bread is not completely frozen until below −70°F (−57°C). Grosse (1966) observed that soap bubbles remained elastic at temperatures down to −22°F (−30°C).

Nuclear Magnetic Resonance (NMR) has been used for the quantitative determination of water dispersed in solids (Miller *et al.*, 1963; Conway *et al.*, 1957). NMR measures the total number of hydrogen atoms present. However, hydrogen atoms of solids, because their molecular motion is restricted, exhibit broad resonance lines while hydrogen atoms of liquids give sharp peaks (Dyer, 1965). Because of this large disparity, it is possible to adjust the NMR instrument to consider only the signal coming from liquid (water) and ignore that from solid (ice, protein, etc.) (Varian Associates, 1966). Thus, Sussman *et al.* (1966) used high resolution NMR to determine the quantity of liquid water in cod muscle at temperatures as low as −50°F (−45°C).

EXPERIMENTAL

THE PRESENT WORK was done with a PA-7 Wide-Line NMR Process Analyzer (Varian Associates, Inc., Palo Alto, California) equipped with an integrator V-4221, a V-4540 variable temperature controller and a PA-7 2-ml variable temperature probe.

A low protein (6.7%) flour obtained by conventional milling of soft red winter wheat followed by air classification was used. Its moisture content was about 10%. This was increased to various levels by cooling the flour to 0°F (−18°C), adding a weighed amount of finely crushed ice, blending in a precooled osterizer for 2 min, transferring to a sealed bottle and allowing to warm to room temperature. For dough of high moisture content with a consistency allowing easy mixing, water was simply added to the flour and the mixture agitated until homogeneity was achieved. Both methods gave the same results.

Fig. 1 shows the sample tube assembly. The outer tube, 10.75 mm ID × 117 mm, was fitted with a glass insert tube 10 mm OD × 3 cm and a capillary tube as shown. The flour or dough sample was placed in the insert tube and the sample tube was assembled; the entire assembly was weighed on an analytical balance before and

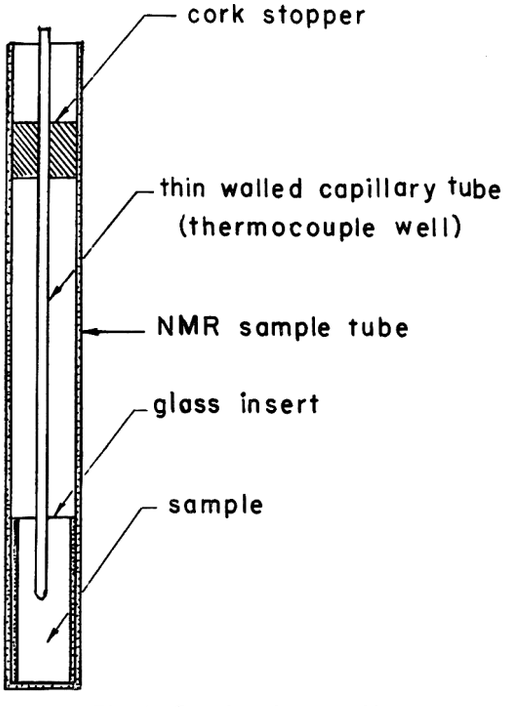

Fig. 1. Sample tube assembly.

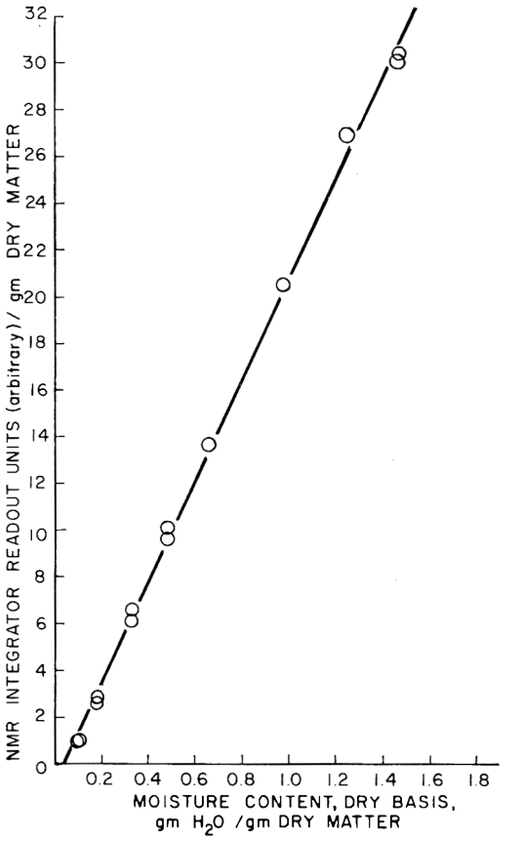

Fig. 2. Calibration curve showing the moisture content of wheat flour and its dough as measured by a wide-line NMR spectrometer.

after this to obtain exact sample weight, about 2 g. All samples were then frozen. When the instrument was available, a given sample was brought to room temperature and the sample tube placed in the instrument. NMR readings were then taken after successive exposure to lower temperatures.

When the sample was placed in the temperature-controlled probe of the instrument, a copper-constantan thermocouple was inserted into the capillary tube to measure sample temperature to ±0.5°F (±0.3°C). The thermocouple was removed every time an NMR sweep was made and then replaced until the next temperature equilibrium had been attained. This obviated electrical interference by the thermocouple metal without disturbing the sample.

Moisture content of samples under 30% was determined by the AOAC vacuum oven method (1955). Determinations at higher moisture contents were made by lightly pressing about 10 g of sample in a folded 8-in. square of filter paper and drying to constant weight in a vacuum oven at 27 in. Hg and 100°C (Mannheim *et al.*, 1957).

RESULTS AND DISCUSSION

FIG. 2 SHOWS A CALIBRATION CURVE of NMR signal as a function of moisture content of the sample at room temperature. The ordinate was calculated by dividing the NMR signals obtained by the weight of dry matter in the sample. The calculated regression line in Fig. 2 intercepted the abscissa at 0.04 g water per g dry matter, which means that a flour sample with a moisture content of 3.48% should give a zero NMR reading at the instrument sensitivity used. A sample freeze dried to 3.2% did show a zero reading.

In determining the effect of sample temperature on NMR reading, two sweeps were made after the temperature remained constant for about 5 min and the average of these two readings was calculated. The line width of the signals was approximately one gauss. No significant change in the line width of the signals was observed with decreasing temperatures down to −58°F (−50°C). Sussman *et al.* (1966) found an increase in line width with decrease in temperature from 0°C to −20°C.

Fig. 3 shows the signal obtained expressed as average integrator readout divided by the weight of dry matter present. At temperatures above the freezing point, no change in NMR signal could be observed with a change in temperature. At 28°F (−2°C), a very sharp break occurred; the NMR signal for each sample decreased to a low value.

As the temperature was further dcreased the NMR readout for all the samples fell on the same gently sloping curve. Thus, flour samples of different moisture content (Fig. 3) showed the same amount of liquid water at a

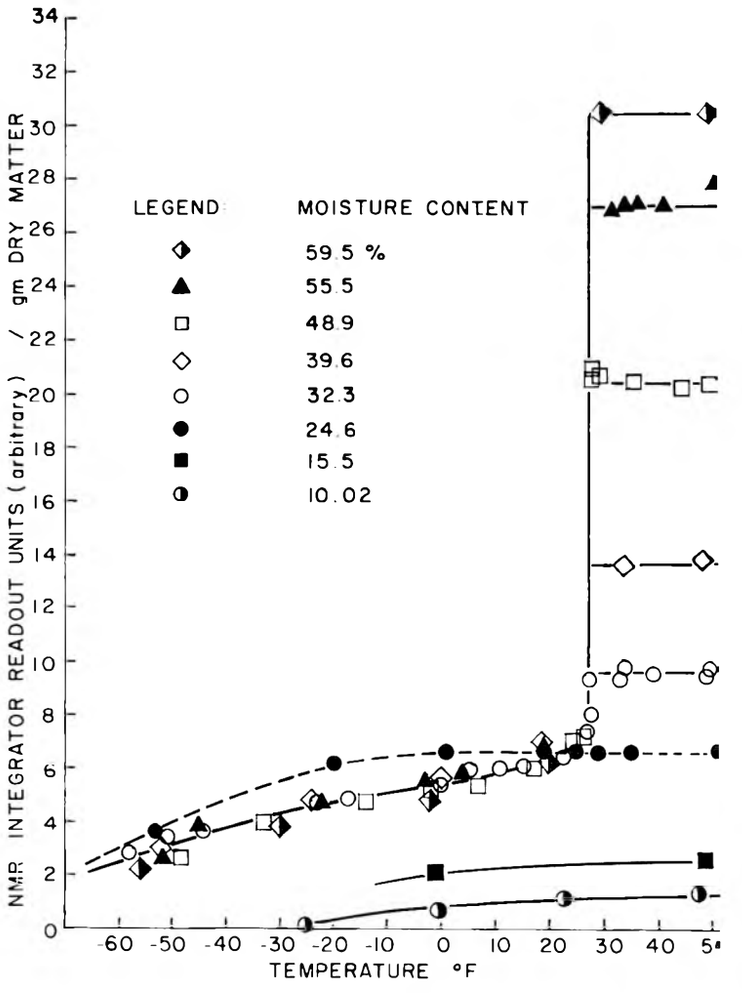

Fig. 3. The liquid water content of wheat flour and its dough at temperatures above and below the freezing point as measured by a wide-line NMR spectrometer.

given sub-freezing temperature. It was therefore concluded that the amount of water bound by a given weight of dry matter is independent of total moisture content.

Another interesting feature of Fig. 3 is the curve for flour with 24.6% water (broken line). This sample was inadvertently hydrated to the moisture level at which the sharp break in the curve was obtained. It it assumed that no ice nucleation occurred at this point. Thus, crystallization of bound water was prevented (supercooling) until a temperature of −10°F (−23°C) was reached. The same trend can be seen for samples with 15.47% and 10.02% moisture. Pichel (1965) observed the same phenomenon in the freezing of egg whites; the water content below which no ice formation occurred was 29%.

A strong liquid water signal, about ⅓ that at 28°F (−2°C), was still observed at −58° (−50°C), the lowest temperature reached during this experiment. Extrapolation of the curve to zero signal shows that the signal would be expected to vanish at about −85°F (−65°C). Similar extrapolation of the ice curve for bread (Mannheim *et al.*, 1957) also showed −85°F (−65°C).

Bound water content

Obviously, not all the bound water is held with an equal force since there is a gradual decrease in liquid water as the temperature is lowered. Therefore, any temperature in this range may be chosen for the NMR reading but it must be specified in the definition. The bound water content may be defined as the water equivalent to the NMR signal (calibration curve) at 0°F (−18°C). It might be pointed out that the curve (Fig. 3) was still quite flat at 0°F (−18°C); the range from +5°F (−15°C) to −5°F (−20°C) corresponded to 0.30 to 0.28 ± 0.01 g water per g dry matter so neither choice of temperature nor temperature control are really crucial.

The average NMR reading at 0°F (−18°C) for the samples with moisture contents above 24.6% was 5.27 with a standard deviation of 0.29 units. This corresponded to a bound water content of 0.29 ± 0.01 g water per g dry matter. A value of 0.286 was obtained for dough (10.1% protein flour) by Vail *et al.* (1940), who used the freezing point depression technique.

This use of NMR is proposed as a new method for measuring bound water. The great disadvantage is cost of buying and maintaining the instrumentation. Otherwise the method is accurate, rapid and nondestructive of sample. Furthermore, in contrast to all other quantitative methods, the NMR signal gives a direct reading of bound water.

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PATRICIA H. POHL, ANDREA C. MACKEY and BARBARA L. CORNELIA
Home Economics Research, Agricultural Experiment Station, Oregon State University
Corvallis, Oregon 97331

Freeze-Drying Cake Batter for Microscopic Study

SUMMARY—A method is reported for preparing cake batter for microscopic study through the technique of freeze-drying, followed by fixation and staining of the fat with osmium tetroxide, infiltration with paraffin, and sectioning of the freeze-dried batter. Microscopic examination reveals that a cake batter is an emulsion of fat in an aerated aqueous phase. The fat particles are irregularly "globular" shaped droplets dispersed throughout the aqueous starch-protein system. In like manner, the air bubbles are dispersed in the batter. They are not incorporated in the fat particles, but are instead distributed throughout the aqueous phase.

INTRODUCTION

THIS PAPER REPORTS a method whereby cake batter may be prepared for microscopic study through the technique of freeze-drying, followed by fixation of the fat, infiltration with paraffin, and sectioning of the freeze-dried batter for study.

The most commonly used procedure in preparing a batter for microscopic study is that of compressing and spreading a drop between a microscope slide and cover glass. For lack of other methods available, the possible physical changes brought about by the preparation of such a "smear" have of necessity been ignored. The most obvious flaws in this method are inability to control the degree of compression, danger of cover glass side-slipping, thus distorting the structural features of the batter, and the action of surface forces imposed by compression between glass surfaces.

The search for methods other than the "smear" is illustrated by the reports of Burhans *et al.* (1942) and Sandstedt *et al* (1954). These studies on bread dough attempted to eliminate doubts inherent in the "smear" technique and to gain additional information.

Relationships between the microscopic structure of batters and doughs and the quality of the resulting baked product have been indicated in studies such as those of Carlin (1944), Jooste *et al.* (1952), and Hunt *et al.* (1955). The quality of the baked product has usually been assessed on its volume, grain, and taste panel response.

The fluidity and plasticity of batters and doughs pose problems in devising ways to study structure. No true cellular structure exists, such as that found in plant and animal tissues. Structural relationships among ingredients are achieved through manipulation, during which process hydrophilic ingredients absorb water, fat is distributed, air is incorporated, and other changes take place. The structure is often fragile and transient, subject especially to factors that alter surface forces, such as time, temperature, and agitation.

Fast freezing and dehydration while in the frozen state has proved to be a satisfactory method for fixing the structural elements of batter. Histological freeze-drying allows the dehydration of tissues without subjecting them to contact with dehydrating fluids. It is based upon the physical phenomenon of sublimation whereby ice in the tissue passes directly from the solid to the vapor state. In most cases the solute remains evenly dispersed as the frozen solvent sublimes. As stated by Flosdorf (1949) the residue is porous and occupies essentially the same space as in the original material.

PROCEDURE

CAKE BATTER WAS FROZEN RAPIDLY to immobilize the structural elements and was maintained at low temperatures during removal of most of the water. Following removal of water, fixing and staining of fat was accomplished by exposing the batter to the vapors of osmium tetroxide (OsO_4) (Gray, 1954). The heavy osmium is adsorbed by the fat, thus protecting it from being dissolved by its usual solvents (Jooste, 1951). Fixation is accompanied by brown to black staining of fatty constituents. Other organic materials are also affected by osmic acid, but dark staining appears to be typical of fats (Bahr, 1954).

When the batter was dehydrated and the fat fixed, it was possible to infiltrate with paraffin and prepare sections for microscopic study.

Technique for freeze-drying

Freeze-drying was carried out using a histological freeze-drying apparatus (Model fc-11, Scientific Supplies Corporation, Cambridge, Mass.). This equipment consisted of a horizontal glass manifold with ground glass connections for two lyophil tubes and a condensor trap. Each lyophil tube can hold a perforated metal cassette assembly which segregates up to ten tissue samples. A boat containing a chemical desiccant was inserted through a ground glass connection near the condensor trap. Connected to the system was a high vacuum pump to draw moisture to the trap which was packed in dry ice and acetone.

Each lyophil tube was set in a Dewar flask and corked temporarily to minimize moisture condensation. To the Dewars were added a layer of crushed ice, 200 g of dry ice, additional crushed ice sufficient to fill the Dewar, and 70 ml saturated solution of calcium chloride (Fig. 1).

A minute drop of cake batter was frozen in a bed of crushed dry ice. Samples approximately one millimeter in diameter were chipped from the frozen batter and transferred directly to coded cassettes, also at dry ice temperature. The cassettes were placed in holders which were then lowered into the prepared lyophil tubes. The lyophil tubes were again corked. When all samples had been prepared in this manner, the lyophil tubes in the Dewar flasks

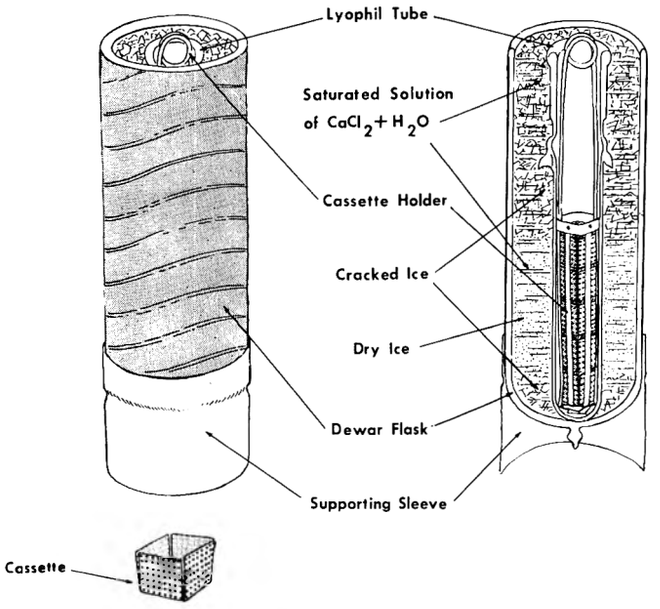

Fig. 1. Equipment used for freeze-drying.

were attached to the manifold of the freeze-drying apparatus and the system was evacuated.

The dehydration process was timed from one hour after the pump was turned on; this allowed the pressure to become established between 0.01 and 0.001 mm mercury. Dehydration was carried out for 48 hr under vacuum, starting at the temperature of dry ice (−72°C). A very gradual rise in temperature occurred until the temperature of the lyophil flasks was 0°C.

The cassette assemblies were transferred to a jar containing calcium sulfate to prevent moisture from condensing on them. The assemblies were then lowered into a glass weighing jar which contained 0.5 g of osmium tetroxide crystals. The covered jar was refrigerated at 6°C for 24 hr, then was left at room temperature for 2 hr. During this interval the fat was fixed by the osmium tetroxide, thus immobilizing it and protecting it from being dissolved in subsequent steps of the procedure. In addition to being fixed during exposure to osmium tetroxide vapors, the fat was stained black. This made it possible to clearly distinguish the fat particles when sections of the batter were viewed under the microscope.

At this point the samples were removed from the cassettes and were transferred to vials containing melted paraffin (Fisher's tissuemat, melting range 60° to 63°C) and tertiary butyl alcohol in a ratio of 1:2. The vials were placed under 15 in. Hg vacuum at 63°C and were allowed to remain overnight. The samples were transferred to vials half-filled with fresh paraffin, a thin layer of paraffin was poured over them, and they were returned to the vacuum oven, where infiltration was continued at the same temperature and the same pressure. Paraffin changes were made after 2 hr and three times thereafter at hourly intervals. One hour after the final paraffin change the samples were embedded in fresh paraffin in a plastic tray such as is used for making miniature ice cubes of ½-in. square. This was returned to the vacuum oven for another hour.

After the tray had been cooled at freezer temperature (−18° C), the paraffin surrounding each sample was trimmed until it could be inserted directly into the block holder of a rotary microtome. Sections 15 μ thick were cut. If brittleness caused the sections to crumble, the cut surface of the dehydrated, fixed batter sample was painted with a 5% solution of glycerin in 70% alcohol or 4% formalin solution, any excess was removed by blotting, and sectioning was resumed. Two drops of Haupt's adhesive were smeared on each slide, the sections were transferred to these slides, and enough 4% formalin was dropped onto the slide surface to cause the sections to float. The slides were then warmed at 42°C for 3 or 4 min. Excess formalin was removed by touching a tissue to the edge of the slide. The slides were left on the warming plate to dry overnight.

The paraffin was removed by immersing the slides for 5 min in a coplin jar filled with xylene, followed by immersion in a second jar of xylene for 5 min, or longer if necessary to remove the paraffin. The bottom of each slide was dried with a tissue, and the slide was returned to the warming plate until all the xylene had evaporated. Cover glasses were mounted with balsam.

RESULTS AND DISCUSSION

When prepared by the freeze-drying technique, a cake batter is seen to be an emulsion of fat in an aerated aqueous phase. The fat particles are irregularly "globular" shaped droplets of various sizes dispersed throughout the aqueous starch-protein system. In like manner the air bubbles are dispersed in the batter. They are not incorporated in the fat particles, but are instead distributed throughout the aqueous phase (Fig. 2).

This type of structure was demonstrated repeatedly for batters made with many different household shortenings.

The concept held for so long in which air bubbles are surrounded by fat, poses difficulty in envisaging the movement of water vapor and carbon dioxide gas through the fat barrier to the enclosed air bubbles, or the coalescence of air bubbles at room temperature as noted by Handleman (1961). In contrast, the distribution of semi-globular particles of fat distinct from the air bubbles which are dispersed throughout the aqueous phase, would enable moisture and carbon dioxide to migrate readily to the air bubbles.

Except for sponge type cakes, the presence of fat or emulsifier or both appears to be necessary for the retention of air that is incorporated during mixing. Valassi (1956) demonstrated that when no fat or emulsifier was present, the incorporated air bubbles coalesced rapidly and formed larger bubbles. The volume of cakes baked from these batters was small, indicating a loss of air from the batters. When fat was added, the viscosity of the batters increased and aeration was much improved. When emulsifier or emulsifier plus fat was added, aeration of the batters was greatly increased. In her study, aeration of batters was least when fat and emulsifier were omitted from the formula, as judged by specific gravity of the batters, intermediate when fat was used, and greatest when emulsifier was added to the formula.

In his discussion of the mechanics of the leavening process, Bailey (1945) says that it appears that pockets

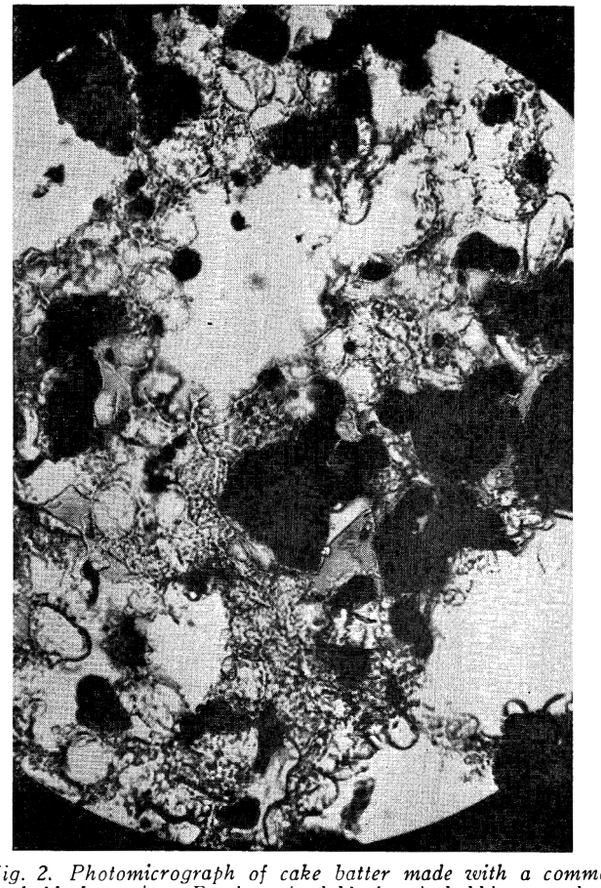

Fig. 2. Photomicrograph of cake batter made with a common household shortening. Fat is stained black; air bubbles are clear; aqueous phase is lacy in appearance and starch grains may be clearly seen.

of gas tend to form at the points where there are particles of fat. The development of a gas pocket requires a cleavage of the dough mass, and it is reasonable to assume that cleavage will most readily occur along the fat boundaries.

Following Bailey's idea, it may be postulated that during mixing, air is incorporated and trapped at cleavage planes occurring at the aqueous/fat interface; or, as aerated fat is emulsified in the aqueous phase, the air is forced outward to the aqueous/fat interface.

CONCLUSIONS

REPORTS IN THE LITERATURE suggest that cake batters are oil in water emulsions, but the evidence has been indirect. Electrical conductivity studies and compatibility of oil or water-soluble dyes with the batter have contributed most confirmation to this theory. It has never been demonstrated visually. The freeze-dry method in which batter structure is immobilized and fixed for study makes it possible to conclusively demonstrate the oil in water structure of cake batter.

The primary structure is an aerated matrix of hydrophilic constituents in which fat particles are dispersed.

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R. M. REEVE and M. S. BROWN
Western Utilization Research and Development Division, Agricultural Research Service
U. S. Department of Agriculture, Albany, California 94710

Histological Development of the Green Bean Pod as Related to Culinary Texture. 1. Early Stages of Pod Development

SUMMARY—The basic pattern of tisue origins is well established in the bean pod when the unopened flower bud is less than 3.0 mm long. An outer epidermis (protoderm) with many hair-like outgrowths forms the outermost tissue of the young pod. A subjacent layer one cell deep forms a hypodermis. Inwardly, a zone 8 to 10 cells deep of enlarging and vacuolating cells forms the young outer parenchyma of the growing pod. Within the innermost half of this zone, the meristematic stage of vascular tissues (procambium) is differentiated.

An inner epidermis (protoderm) and an ill-defined hypodermis line the pod cavity. The young inner epidermis is continuous with the outer epidermis of the young seed (ovule). Cell divisions in the inner epidermis and its hypodermal layer initiate an inner parenchyma and fiber sheath, respectively, approximately at the end of flowering. The inner parenchyma becomes extensive in early post-floral stages. At the same time, the initials of the fiber sheath become sharply differentiated by cell elongation at a 45° angle to pod length, and assume the characteristics of young fiber cells (sclereids).

Cell divisions cease first in the outer parenchyma, and most post-floral growth of this tissue is by cell enlargement. Cell divisions persist through early post-floral growth in the inner parenchyma, young fiber sheath, procambial tissues and outer epidermis. These differences in origin and rates of early development of pod tissues foreshadow marked differentiation and structural specializations that are significant to textural qualities at culinary maturities.

INTRODUCTION

ALTHOUGH THE CULINARY QUALITIES of green beans have been well investigated from the viewpoint of processing, relatively little attention has been accorded the relationships of tissue structure to texture problems. Apparently this has been due to the paucity of information concerning the developmental sequence and differentiation of the various pod tissues. Nearly all published botanical literature on the development of the legume pod has emphasized the morphological interpretation and evaluation of the legume as a fruit structure (Esau, 1965; Hayward, 1938). In such studies the development of the vascular bundles, or conducting system, has been described in detail.

Very little published information exists as to origins and development of the soft, parenchyma tissues constituting most of the edible pod. Such information should be useful in understanding the various kinds of tissue damage that often result when green beans are frozen (Brown, 1967; Wolford *et al.*, 1965) or are otherwise processed.

The time and extent of development of the vascular bundles and other fibrous tissues in the pod likewise are significant to the culinary textural qualities of green beans. The cell walls of the fibrous tissues thicken greatly and become lignified on maturity, forming the tough strings and the papery layer. Although these tough, fibrous tissues have been minimized in development of "stringless" varieties (Currence, 1930; Kooiman, 1931; Stark *et al.*, 1942), their composition and time of maturation vary according to variety and environment (Kaldy, 1966; Singh *et al.*, 1966). In addition, changes induced by post-harvest temperatures and processing treatments suggest need for further consideration of the structural distinctions between tissues within the pods (Kaczmarzyk *et al.*, 1963; Watada *et al.*, 1966a,b).

Even the cell walls of soft, parenchymatous vegetable tissues tend to toughen when preserved by dehydration because of increased cellulosic crystallinity (Shimazu *et al.*, 1965; Sterling *et al.*, 1961). Such toughening can be pronounced in fibrous tissues because they are characteristically small-celled and consequently contain a greater amount of cellulosic wall material per unit volume of tissue than does the soft, large-celled parenchyma.

There is little histological information comparing the development of these various tissues. For example, the descriptions of Stark *et al.* (1942) of the fiber sheath do not include the concomitant development of the soft, parenchyma tissues. Such information should aid in understanding the relationship of tissue organization to various textural defects in processed beans. Because these tissues arise at different times during pod growth, it is further pertinent to consider their full course of development. This first report describes the origins and early growth of pod tissues, beginning with their differentiation in the bean flower. This approach also permits some simplification of conflicting terminology often applied to bean pod tissues. A second paper will compare tissue characteristics within pods at culinary maturities with particular reference to texture problems.

MATERIALS AND METHODS

PODS REPRESENTING ALL STAGES of development, from those in floral buds to the onset of drying and ripening, were collected from several common varieties of beans, including California small white, California red, and pinto in addition to "stringless" varieties of both round- and flat-shape pods. Floral samples and quarter-inch-long pieces of young pods were killed and fixed 24 hr either in an aqueous chromium trioxide, acetic acid and formaldehyde solution (CRAF) or in formalin and acetic acid in 60% ethyl alcohol (FAE). These were then dehydrated, infiltrated and embedded in paraffin by standard procedures. Some of those killed in the aqueous reagent were

embedded either in Carbowax, according to the method of Riopel *et al.* (1962), or in a mixture of Carbowax and a polyvinyl acetate resin, by the method of Reid *et al.* (1966).

Sections were cut serially from embedded samples with a rotary microtome at thicknesses of 7 to 15 μ, depending on cell size and pod age. Mounting and permanent staining was accomplished for paraffin embedded sections by an adaptive schedule to suit maturity conditions, using Delafield's hematoxylin, safranin and fast green (Reeve, 1948). Sections from Carbowax-embedded samples were stained with a Delafield's hematoxylin solution. Some half-grown and older pods were sectioned in the fresh condition at about 30 μ with a sliding microtome. These were stained with a dilute aqueous solution ($< 0.1\%$) of methylene blue for examination.

RESULTS

Origin of the pod

Some consideration of the origin of the bean pod serves to clarify terms applied to different tissues and structural features. The bean pod begins as an open crescentic primordium growing upward from the growing point, or floral apex, which produces the flower. This early formation is similar to that of other leguminous pods (Goebel, 1887; Guard, 1931; Newman, 1936). The open primordium soon closes, from basal portion upward, forming a conduplicate carpel.

Ovules (young seeds) develop in separate rows along two placentae of the ovarian portion of the carpel. Morphologically, the conduplicate carpel represents a leaf folded along its midrib, with its margins fused and with the two halves of its upper surface facing and forming the inner carpel surface (Bailey *et al.*, 1951). Thus, the inner epidermis of the pod represents the upper, or ventral, epidermis of the leaf, and the outer epidermis of the pod represents the lower, or dorsal, epidermis of the leaf. The epidermises of both carpel and leaf are thus continuous. It is also appropriate to further define the pod edges on which the ovules arise as the ventral suture, each edge possessing a ventral vascular bundle. The opposite or midrib side of the pod is likewise considered to be dorsal and the main vascular bundle there is the dorsal bundle (Bailey *et al.*, 1951; Woodcock, 1934).

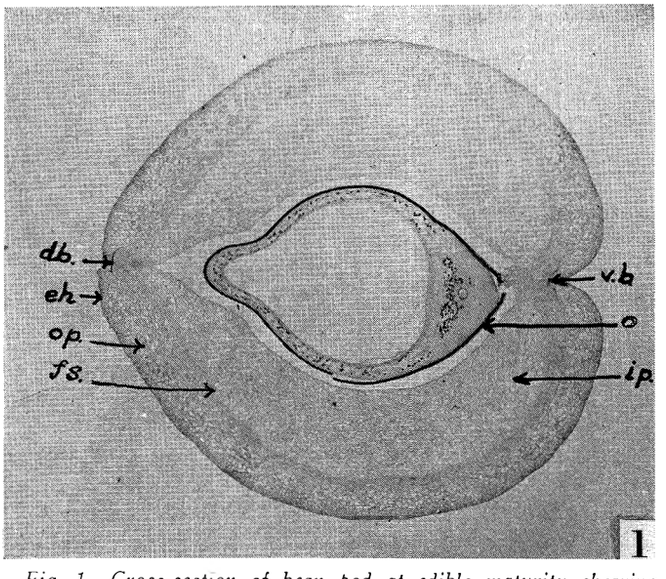


Fig. 1. Cross-section of bean pod at edible maturity showing extent of inner parenchyma at site of a seed attachment, × 6; db—dorsal bundle, eh—outer epidermis and hypodermis, fs—fiber sheath, ip—inner parenchyma, o—ovule or young seed, op—outer parenchyma, vb—ventral bundles.

Despite these similarities, differences in the pods of common legumes become evident during the course of their development. For example, the wall of the pea pod remains thin by comparison with the fleshy green bean pod. This difference, as described later, is due in part to a prolongation of the cell division phase of growth along the inner surface of the very young bean pod. The same sort of growth gives rise to the inner pod tissue of the lima bean, often referred to by the trade as "ice" and used as a criterion of harvest maturity for some varieties of lima beans. In the green bean pod this growth phase gives rise to the soft, translucent tissue comprising the inner portion of the pod at prime maturity for processing (Fig. 1). The histogenesis of this tissue and its differentiation from other pod tissues will be described first.

Floral stages of growth

The basic pattern of tissue development in bean pods is clearly evident in flower buds several days before actual flowering. Fig. 2 illustrates this degree of tissue organization in cross-sectional view at about the time the unopened petals have formed a bulbous structure about 2.5 to 3.0 mm long. The outer epidermis, or dorsal protoderm, of the carpel that forms the young pod is well defined and possesses many epidermal hair outgrowths. Early differentiation of the outer parenchyma zone of the young pod is evident by the degree of cell enlargement and more pronounced vacuolation of its cells. An inner parenchyma zone has not yet been formed.

The immediate sub-epidermal layer between outer parenchyma and the young outer epidermis is definable by cellular characteristics and position (Fig. 3). In addition, it can be seen that the cells of the innermost layer (Fig. 2 and 3) appear nearly isodiametric in cross-section and their contents are dense by comparison with those of the young outer parenchyma. This innermost layer is the inner protoderm and respresents the meristematic phase of the upper or ventral epidermis of a leaf. At the base of Fig. 2 it can be seen that the outer and inner protoderm of the very young carpel (or pod) are continuous, fusing along the ventral suture on each side of which the ovules are borne.

Cells immediately interior from the inner protoderm are of similar appearance, but form a less defined layer (Fig. 3). They represent a meristematic phase of an inner or ventral hypodermis. In addition, early procambial phases in the development of lateral vascular bundles of the pod are evident in the inner regions of the young outer parenchyma. Ventral and dorsal carpellary vascular bundles containing a few protoxylem elements also are present in the suture and midrib areas, respectively (Fig. 2), in a comparatively advanced stage of differentiation. All of the vascular tissues of the pod walls arise within

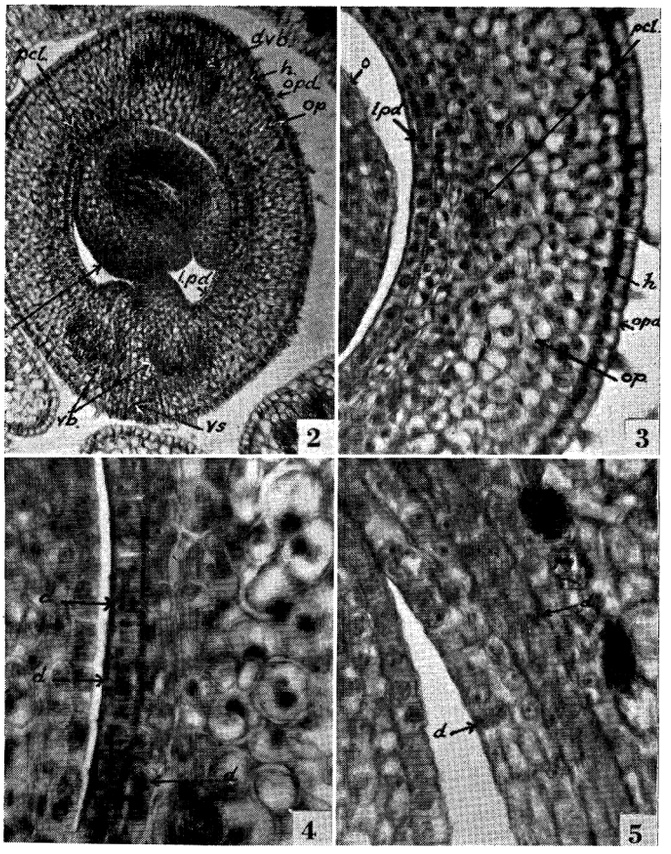


Fig. 2–5. Sections showing early floral stages in carpel development. (2) Carpel from young pod ca. *2.5–3.0 mm long, × 102. (3) Carpel wall at higher magnification, same age, × 273. Note enlargement and vacuolation of outer parenchyma cells; dvb—dorsal vascular bundle, h—hypodermis, ipd—inner protoderm (or epidermis), o—ovule, op—outer parenchyma, opd—outer protoderm, pcl—procambium of lateral bundles; vb—ventral vascular bundles, vs—ventral suture. (4) Inner portion of wall of carpel of same age × 407. Note nuclear division "d" in the inner protoderm and underlying cells. (5) Inner portion of wall of young carpel at time of flowering, × 407. Note nuclear divisions "d" in initials of the fiber sheath and inner parenchyma, also the demarkation between these tissues.*

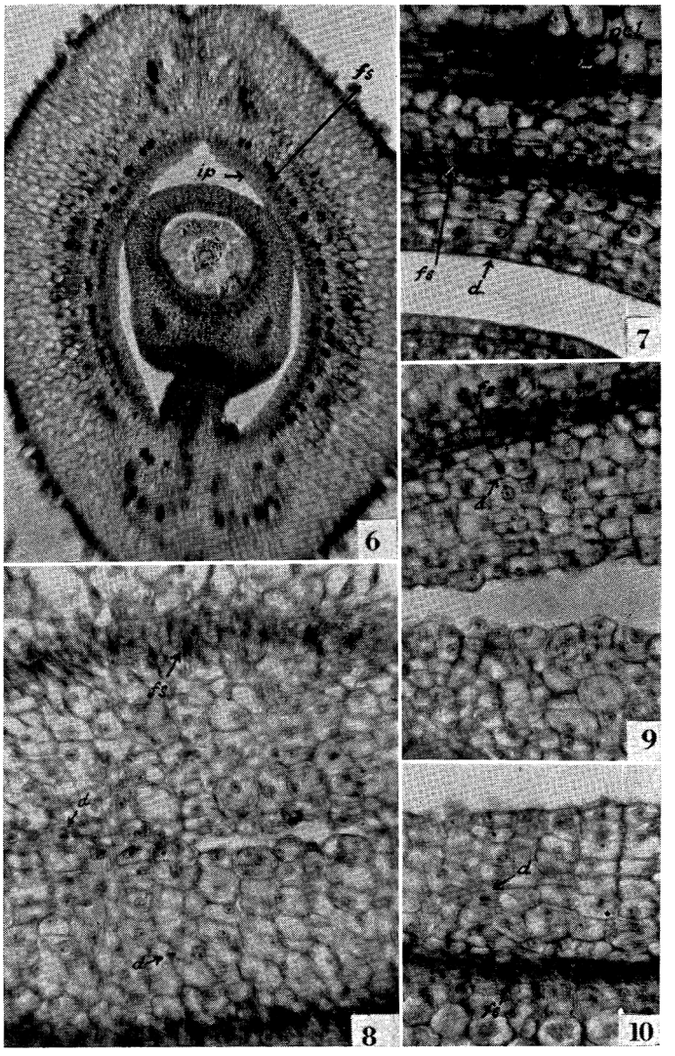
the young outer parenchyma during floral and early post-floral growth. Vascular bundles are never developed in the inner parenchyma zone.

A very active phase of growth by cell division is initiated in the inner protoderm and the subjacent hypodermal layer when the flower bud is still quite small. This is shown (Fig. 4) by nuclear divisions in the young pod of a flower bud about 4 or 5 mm in length. Derivatives from these divisions eventually form the fiber sheath and the inner parenchyma. Shortly after the flower has opened and the young pod, exclusive of its stylar portion, is about 5 mm long, both the inner protoderm and its subjacent hypoderm have become multilayered (Fig. 5) by cell divisions in planes parallel to the inner surface.

These divisions occur at a greater rate in the derivatives of the inner protoderm than in the hypodermal derivatives. Some divisions perpendicular to the inner surface add cells to allow for circumferential growth in both layers. Similar divisions also contribute to growth of the outer protoderm. In the meantime, considerable cell enlargement and occasional division occurs in the young outer parenchyma tissue. By the end of flowering the young pod has more than doubled in cross-sectional dimensions over the first stage described here (compare Fig. 2, X102, with Fig. 6, X68).

Post-floral growth

Further differentiation between derivatives of the inner protoderm and hypoderm layers takes place rapidly. As shown in Fig. 5 and 6, hypodermal derivatives tend to elongate in directions circumferential to the young pod, even in late floral stages of growth. By the time the young pod, excluding its stylar part, is about 10 mm long the



Figs. 6–10. Stages in early post-floral development of young carpels. (6) Cross-section of young carpel at site of ovule attachment and at end of flowering × 68. Note the early differentiation of fiber sheath (fs) and inner parenchyma (ip) initials. Dark areas just inside the young fiber sheath are outer parenchyma cells rich in phlobophenes (condensed tannins). (7) Very slightly older stage than in (6) and showing an inner portion of the young carpel wall, × 203. d—nuclear division in surface cell of inner parenchyma, fs—young fiber sheath, pcl—procambium of lateral bundles. (8–10) Cross-sectional views of inner portions of a young carpel with pod about 2 cm long, × 203. d—nuclear division, fs—young fiber sheath. (8) Near distal (stylar) end of carpel cavity, showing closure of inner parenchyma. (9) Just beyond site of ovule attachment, nearing closure of ovule or seed cavity; note elongation of fiber sheath initials. (10) Near site of ovule; note tiering of inner parenchyma initials.

hypodermal derivatives are distinctly elongate and narrow by comparison with the derivatives from the inner protoderm. Cells of the latter continue to divide in planes both perpendicular to and parallel with the inner surface of the pod cavity (Fig. 7).

Some of the early-formed derivatives from the inner hypodermis appear to contribute to parenchymatous tissue betwen this layer and the lateral provascular bundles in the outer parenchyma. However, most of the ensuing cell division and elongation in hypodermal derivatives appear to occur in planes slightly oblique to the inner surface of the pod. Thus, their appearance in sectional view varies at different cross-sectional levels in the young pod (Fig. 7–10). These derivatives are young sclereids that later become the fiber sheath separating the outer from the inner parenchyma tissues of the pod as shown in Fig. 1 (cf. Stark *et al.*, 1942). Derivatives from the inner protoderm are more extensively developed between the ovules and at the distal and basal ends of the pod cavity than in the pod wall areas opposite the ovules (Fig. 7–10).

Within relatively few days after flowering, elongation of fiber sheath initials is pronounced and appears to be at about a 45° angle to the pod basis. By the time the young pod is from 2 to 3 mm wide, the fiber sheath initials average about 10 or 12 μ in width and 50 to 100 μ or more in length (Fig. 11). "Transition" cells, showing characteristics between the fiber sheath cells and the parenchyma, occur on either side of the young fiber sheath. Most of these transition forms are slightly elongated. They are most abundant on the outer parenchyma side (Fig. 11).

Other closely associated cells of a more parenchymatous nature are slightly elongated at right angles to the fiber sheath cells. Stark *et al.* (1942) have described the fiber sheath as "inner mesocarp" and state that the cells are oriented lengthwise "at an oblique angle to the length of the pod, pointing in the direction of the stylar end from the dorsal suture . . . curved, following the curvature of the carpel from suture to suture." Observations in the present studies confirm this.

When the young pod is about 2.5 mm wide, or about 3.5 mm in dorsi-ventral dimension, cross-sectional views show the inner parenchyma to consist of 10 or more fairly regular tiers of cells in the pod sides opposite the ovules (Fig. 12). At this stage the inner parenchyma is more extensive than outer, earlier-differentiated parenchyma. The inner surface of the inner parenchyma consists of small cells, and the young fiber sheath cells form a distinct boundary of very narrow cells about 3 or 4 deep between the inner and outer parenchyma tissues.

Some cell divisions continue to take place in the young inner parenchyma for a short time while cell enlargement proceeds. By the time the young pod is about 5 mm wide, or 6 mm in dorsi-ventral dimension, the inner parenchyma may be 30 to 40 or more cells deep and the tiering, due to cell divisions parallel to the inner surface, is no longer obvious (Fig. 14). In areas between young seeds, the inner parenchyma has undergone even more extensive growth and the inner surface cells either form a mutual boundary between halves of the pod, or become fused in the more distal regions of the young pod (Fig. 14). Further growth beyond this stage appears to be entirely due to cell enlargement except for occasional cell divisions in the outer epidermis and in the young vascular bundles. The young pod by this time may be about 5 cm in length, depending upon variety and environmental conditions influencing its growth rate.

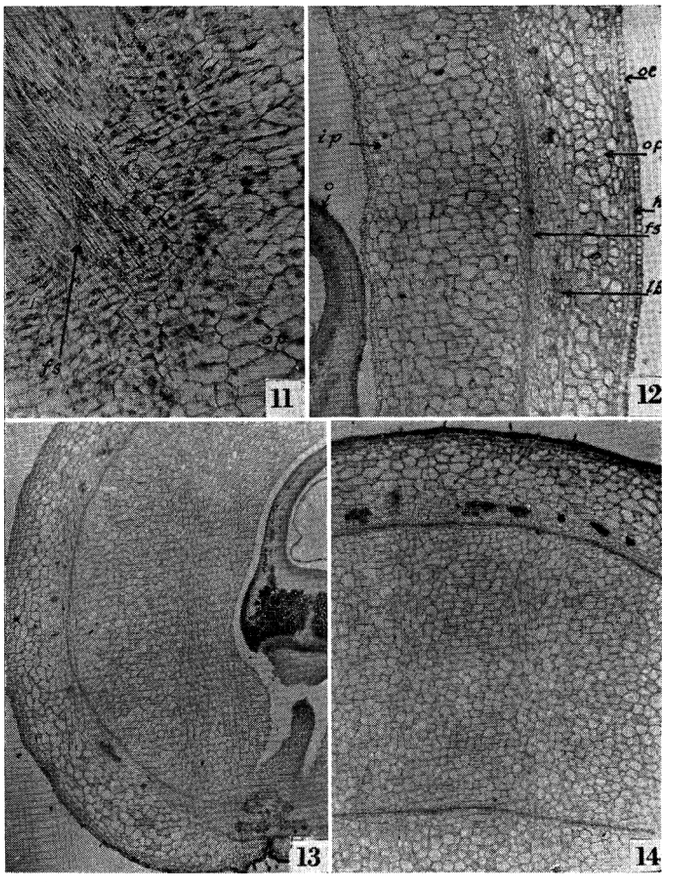


Fig. 11–14, (11). Section from Carbowax-embedded sample cut tangentially to young pod surface and through young, elongating fiber sheath cells, × 102. Young pod ca. *3.0 mm wide; fs—fiber sheath initials, op—outer parenchyma; note some young outer parenchyma cells appear elongated at right angles to fiber sheath initials. (12) Cross-section from young pod about 3.0 mm wide embedded in Carbowax and showing extent of inner and outer parenchyma, × 34. fs—fiber sheath, h—hypodermis, ip—inner parenchyma, lb—lateral bundles, o—ovule, oe—outer epidermis, op—outer parenchyma. (13–14) Cross-sections from young pod about 5 mm wide embedded in Carbowax. (13) × 20 at site of ovule attachment. (14) × 27; between ovules.*

DISCUSSION

THIS IS BELIEVED TO BE the first detailed account of the origins and early development of the major tissue zones of the green bean pod. Earlier publications have described the development and organization of the vascular bundles in legumes, both in relation to carpel evolution (Bailey *et al.*, 1951; Esau, 1965; and Woodcock, 1935) and in relation to "stringiness" of the green bean pod (Joosten, 1927; Kooiman, 1931). A brief, comparative study of the development of the fiber sheath in several varieties also has been published (Stark *et al.*, 1942). The origins and differentiation of the softer, parenchymatous tissues and specialized skin tissues have received little

attention in modern plant anatomy. Such tissues comprise the bulk of the bean pod at edible maturity. They differ in composition as well as in wall structure and morphology, and these distinguishing features further relate to the texture defects encountered in processing. Because of different times of origin and rates of maturation, these tissues also respond differently to environmental factors affecting their development (Kaldy, 1966; Singh *et al.*, 1966).

In addition to the basic pattern of tissue organization well established before flowering, early differentiation of the outer parenchyma cells by enlargement and vacuolation at or before flowering establishes this tissue as histologically older than the other pod tissues, in which growth by cell division continues for a much longer time. These early distinctions initiate the later cell differentiation and specializations that are pronounced by the time the pod reaches edible maturity.

The origin of the inner parenchyma from the inner protoderm of the carpel by cell division is in unique contrast to the early differentiation of the outer parenchyma. Origin of the fiber sheath from cell divisions in the cells immediately subjacent to the inner protoderm, and their subsequent differentiation by cellular elongation, are likewise unique developmental features. This sequence of development appears to differ from observations of Stark *et al.* (1942) who followed pod development only from anthesis onward. They state that the fiber sheath, or "inner mesocarp differentiates from parenchymatous tissues.

"The variety Bountiful exhibits, at the date of anthesis, a single-celled layer, separate and distinct from the tissue on either side, which is the initial of the inner mesocarp. Giant Stringless Green Pod, at the date of anthesis, shows no such prominent separation of tissues, and all cells are parenchymatous and of the same size." On the contrary, it is evident from pre-anthesis stages in present studies that the basic pattern of the tissue origins in the pod is well established when the flower buds are quite small.

Cell size difference between the young outer parenchyma and other tissues in the carpel were likewise evident before anthesis in all varieties studied. Although Stark *et al.* apparently described a later stage than that of initiation of inner parenchyma and fiber sheath, it is possible some varieties may differ as to time of initiation of these tissues, or the difference may relate to growing conditions.

Enlargement and vacuolation of the outer parenchyma cells characteristically occurred very early in all varieties and differed only as to extent, usually being slightly more advanced in flat than in round pods, and in dry seed varieties than in others. Initiation of the fiber sheath by division of cells subjacent to the inner protoderm was characteristic, and differences between varieties were mainly in the rates of their subsequent growth by cell elongation. This variation also could be influenced by growing conditions. The major distinction between varieties with respect to the inner parenchyma was in the greater extent of its development in "stringless" edible pod varieties than in those usually grown for dry beans, and also the more extensive development of this tissue in round-podded than in flat-podded varieties.

A confusion of terminology exists in much of the literture on pod anatomy to which food scientists must refer. Simplification of this terminology is possible by eliminating terms used for submorphological divisions of the carpel or pod wall. For example, on the basis of tissue histogenesis, the inner parenchyma, arising from the inner protoderm, could be defined as a multiple epidermis forming the endocarp of the pod. Winton *et al.* (1935) consider this an inner mesocarp without regard to its origin. The fiber sheath, however, seems reasonably definable as an inner mesocarp, in agreement with Stark *et al.* (1942)—or simply as mesocarp, if taken together with the outer parenchyma. Stark *et al.* consider the outer parenchyma and outer epidermis to be exocarp.

In a stricter morphological sense, the term exocarp is usually applied to the outer epidermis, in keeping with the term endocarp for inner epidermis (Sterling, 1953). However, these distinctions are more artificial than natural and their homology breaks down when applied to other fruits. For these reasons, the inclusive term pericarp seems best to use for all tissues comprising the carpel or fruit wall (Esau, 1965). In the bean pod this includes all tissue from outer epidermis to the cavity lining (Fig. 1). One may then apply the appropriate descriptive terms for the specialized cell types that become differentiated within the pericarp during growth and maturation.

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R. M. REEVE and M. S. BROWN
Western Utilization Research and Development Division, Agricultural Research Service
U. S. Department of Agriculture, Albany, California 94710

Histological Development of the Green Bean Pod as Related to Culinary Texture. 2. Structure and Composition at Edible Maturity

SUMMARY—The different tissues of the green pod exhibit a wide range in cellular structure and composition at edible maturities. Large substomatal spaces and intercellular spaces in outer parenchyma and thick-walled hypodermis contribute to cell separation or sloughing when the beans are cooked. Cells of the outer parenchyma contain numerous starch granules, are thicker walled than the inner parenchyma cells, and usually are not torn as a result of freezing. Cells of the inner parenchyma are thin-walled and form a compact, succulent tissue with only minute intercellular spaces. Slow rates of freezing result in radial cracks often extending into the young fiber sheath lying between the inner and outer parenchyma zones. With more rapid freezing small cracks sometimes appear only in close association with the immature fiber sheath cells.

Prolonged immersion freezing usually results in radial splitting throughout all tissues without respect to differences in structure and composition. In pods overly mature for culinary use wall thickening and lignification of sheath sclereids and sclereid caps contribute to toughness and stringiness. Other histological aspects of texture qualities involve growth environment and postharvest conditions.

INTRODUCTION

THE ORIGINS AND EARLY DEVELOPMENT of pod tissues were described in Part 1 of this study and it was emphasized that the basic pattern of tissue differentiation is very well established in very early stages of flower bud development (Reeve *et al.*, 1968). In brief, the inner soft parenchyma of the pod arises from the inner epidermis or protoderm by cell division before the flower has opened. At the same time, the fiber sheath of over-mature pods arises from cells immediately subjacent to the young inner epidermis. Cells of the outer parenchyma tissues of the pod, already enlarging and becoming noticeably vacuolate before flowering, have virtually ceased to divide at time of flower opening or shortly thereafter. These differences in times of origin and subsequent rates of tissue maturation render the pod susceptible to environmental factors that influence composition, fiber content, and culinary quality at edible maturity (Kaldy, 1966; Watada *et al.*, 1966a,b). The main features of these later differences in structure and composition of the various tissues are described in the present communication, with special reference to freezing damage and other textural qualities.

MATERIALS AND METHODS

HISTOLOGICAL PREPARATION OF SAMPLES by the paraffin and Carbowax techniques were as previously described (Reeve *et al.*, 1968). In addition some sections were cut directly from fresh, maturing pods, using a sliding microtome. Histochemical tests were made principally upon fresh sections. These included the phloroglucinol-hydrochloric acid and the chlorine-sulfite methods for lignin (Jensen, 1962), Bial's orcinol method for pentosans (Reeve, 1946a), and the ferric hydroxamate reaction for esterified pectins (Gee *et al.*, 1959; Reeve, 1959). Preparation of sections from samples representing different freezing treatments was as previously described (Brown, 1967; Wolford and Brown, 1965).

RESULTS

Inner and outer parenchyma

Fig. 1 illustrates the development of a pod at early edible maturity, or about 8 mm in width. The cells of the outer epidermal and hypodermal layers are more

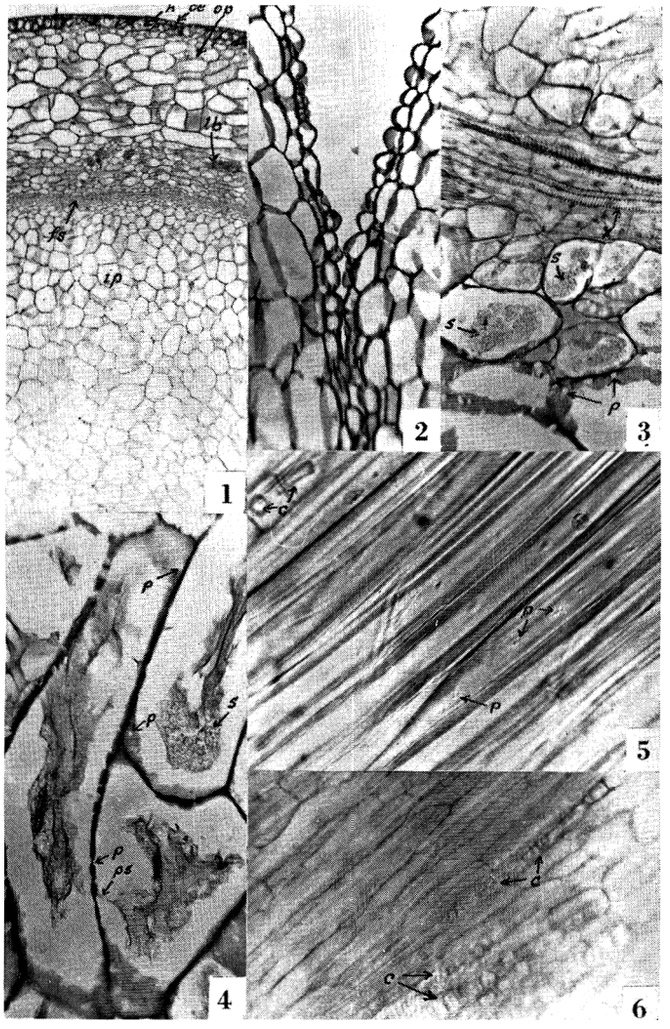

Fig. 1–6. Tissues of bean pod at edible maturities. (1) Cross-section, × 25; pod 8 cm wide, medium maturity; fs—fiber sheath, h—hypodermis, ip—inner parenthyma, lb—lateral bundle, oe—outer epidermis, op—outer parenchyma. (2) Cross-section from Carbowax-embedded sample about medium maturity showing papillate cells of inner surface and their interlocking as occurring at the ends of the pod cavity and dorsally near the seed cavities, × 68. (3) Tangential section through lateral bundle area of outer parenchyma in pod of medium maturity, × 150; p — wall pits, s — starch, x — xylem of lateral bundle. (4) Tangential section through larger outer parenchyma cells showing wall pits (p) in sectional and surface views, × 102. Note protoplasmic strands (ps) near a pit; s — starch. (5) Tangential section through fiber sheath sclereids of a pod at or near end of edible maturity, × 407. Note bordered pits (p) of thickened walls, crystals (c) and tapered ends of sclereids. (6) Tangential section through transition cell area of fiber sheath, × 203. Note crystals (c) and blunt ends of cells.

heavily stained, because of their thicker walls, than the underlying parenchyma tissue. A wide range in cell size and shape is evident in the outer parenchyma with much smaller cells occurring immediately beneath the hypodermis and also more extensively in the area in which the latter vascular bundles of the pod develop.

The cells of the inner parenchyma tend to be more uniform in size and shape, forming a more compact tissue than the cells of the outer parenchyma. In some pods the inner parenchyma cells are on the average smaller than those of the outer parenchyma; in other pods, they tend to be larger. These differences in cell size within a variety probably reflect differences in growing conditions at the time of growth by cell enlargement. Intercellular spaces are uniformly very minute in the inner parenchyma. The cells lining the pod cavity are more variable in shape and do not form a discrete layer. In their natural conditions, as seen both in fresh sections and those cut from Carbowax-embedded samples, many of these innermost cells are papillate at sites opposite the seeds. Where opposite sides of the pod meet, these papillate cells are interlocking, or appear to be fused (Fig. 2).

Chloroplasts are present in the outer parenchyma, appearing very early in pod development, and occur most abundantly in the outer and middle regions of this zone. The inner parenchyma, usually lacking in green pigment, contains no obvious chloroplasts. In some varieties a faint green pigmentation occurs and apparently rudimentary chloroplasts are present.

Very small rounded and sometimes angular starch granules are abundant, particularly in the middle and innermost cells of the outer parenchyma (Fig. 3 and 4). These appear as the pod undergoes rapid elongation before harvest maturity is reached. They are numerous during edible maturity stages and some are present as the pod begins to ripen. Winton *et al.* (1935) consider starch granules to be transistory.

Although starch may disappear in the ripening process, the granules were always found in the present study to be abundant during edible maturity stages of pod development. It is reasonable that the amount of starch present varies mainly according to the conditions that influence photosynthetic rates. A few very small starch granules were observed in the inner parenchyma cells of some pods, but starch storage does not appear to be characteristic of this zone.

The outer and inner parenchyma cells also differ in degree of cell wall development. As reported earlier (Reeve *et al.*, 1968) the outer parenchyma is well differentiated by cell enlargement and vacuolation during floral stages before the inner parenchyma has been initiated. Thus, at edible maturity, the outer parenchyma may be considered histologically more mature than the inner parenchyma. The cell walls of the outer parenchyma are thicker and stain more intensely with Delafield's than those of the inner parenchyma.

Primary pit fields are pronounced and abundant in the walls of the outer parenchyma cells (Fig. 3 and 4). These are very thin, minute, oval areas in the primary walls of plant cells through which protoplasmic strands (plasmodesmata) connect the protoplasts of adjacent cells through a very thin net of cellulosic microfibrils (Esau, 1965). Primary pit fields also are present in the walls of the inner parenchyma cells but are less distinct in stained sections because the walls of these cells are thinner than those of the outer parenchyma. In addition, the compound middle lamellar regions of the walls of the outer parenchyma give a more intense ferric hydroxymate color reaction for pections than do those of the inner parenchyma.

Fiber sheath and vascular bundles

In all pods of young edible maturity examined, the young sclereids of the fiber sheath had not yet developed

any extensive secondary wall thickening. In the younger of these pods the walls of the fiber sheath sclereids were actually thinner than those of parenchyma tissues on either side. Secondary wall thickening of the sclereids was not clearly evident until the pods were in later stages of edible maturity. Characteristic wall thickening was clearly evident when the pods were overly mature and just beginning to toughen (Fig. 5).

The secondary walls of these sclereids possess x-shaped bordered pits. This form of pit consists of a narrow, lenticular gap in the wall of one sclereid oriented at an angle to a similar gap in the well of the adjacent sclereid (Esau, 1965). At maturity these fiber sheath sclereids, usually with tapered ends, range from 15 to 30 μ in width and 300 to 500 μ or more in length. As previously reported (Reeve *et al.*, 1968), they are oriented lengthwise and dorsi-ventrally to the pod at about a 45° angle. This orientation is approximately at right angles to that of the elongated hypodermal cells, to be described later.

Associated with the fiber sheath sclereids is an assortment of shorter elongated transition cells that range up to 50 μ in width and from 100 to 200 μ in length and are oriented parallel with the sclereids. These have blunt to slightly tapered ends and their secondary walls possess bordered pits matching those of the sclereids. Many contain crystals (Fig. 6). These transition forms are most highly developed on the outer parenchyma side of the fiber sheath where they exhibit a wide range in size and shape. Development of transition forms is much less pronounced on the inner parenchyma side of the fiber sheath.

The fiber sheath was less developed, with respect to pod size, in those varieties bred for culinary use as green beans than in those grown for dry seed. This would indicate a prolongation of the more succulent stages of growth in the former, with a comparatively more extensive development of the softer parenchyma tissue. When fully developed, the walls of the fiber sheath sclereids of both types of beans gave positive reactions to lignin and pentosan tests.

Stark *et al.* (1942), using the phloroglucinol test for both lignin and hemicelluloses, reported positive reaction for xylan-araban hemicellulose but negative for lignin. This method, however, may not be as selective as those used here because it depends on heat treatment to distinguish hemicellulose from lignin. In present studies, only faint lignin reactions were obtained in the walls of fiber sheath sclereids of pods approaching culinary overmaturity, but positive pentosan tests were obtained. Secondary wall thickening had only begun in these.

Sclereids also form distinctive caps over both the dorsal and ventral vascular bundles of the pod (Fig. 7 and 8). They are less extensively developed in the pods of varieties selected for "stringlessness." Joosten (1927), Currence (1930), and others have described these tissues in detail with reference to inheritance of stringiness. The mature walls of these sclereids respond positively to both lignin and pentosan tests. Secondary wall thickening in the xylem elements of the vascular bundles are likewise lignified but yield only faint reactions for pentosans.

Much smaller, branching and anastomosing vascular bundles are present in the outer parenchyma of the pod

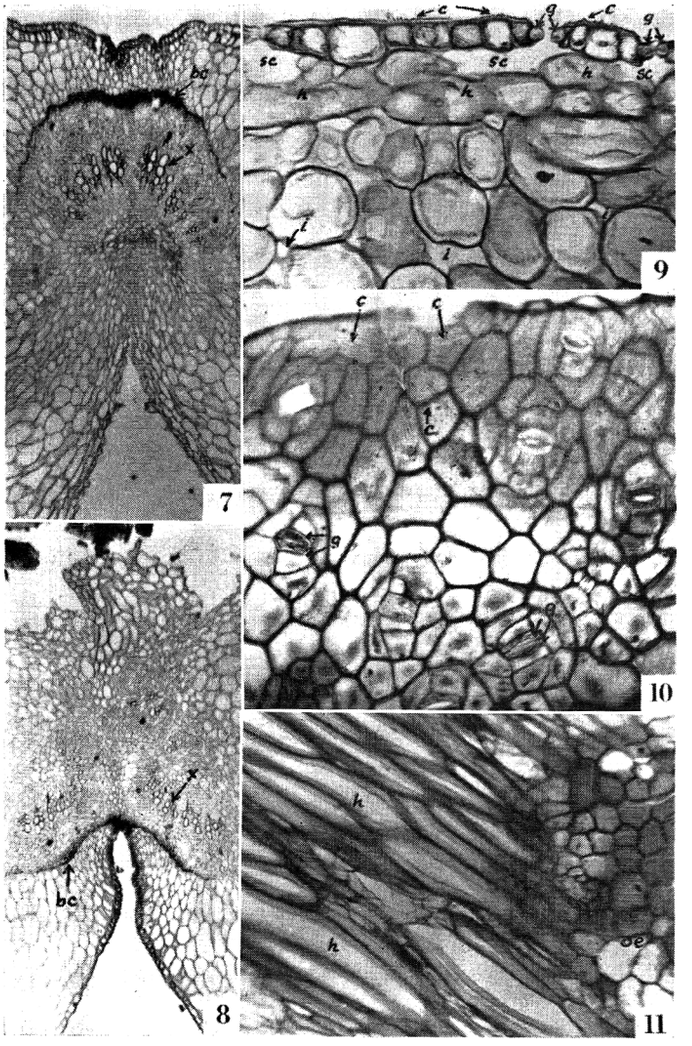

Fig. 7–11. Tissues of bean pod approximately at end of edible maturity. (7 and 8) Cross-sections of dorsal and ventral bundle areas, respectively, showing bundle caps (bc) and xylem (x) of vascular bundles, × 41. (9) Cross-section of outer epidermis and hypodermis of pod, × 136; c — cuticle ridges — guard cells of stomata, h — hypodermis, i — intercellular spaces in outer parenchyma, sc — substomal cavity. (10) Tangential section through outer epidermis and cuticle, × 105; c — cuticle ridges, g — guard cells of stomata. (11) Tangential section through hypodermis of same pod, × 68; h — hypodermal cells (note heavily stained primary and lightly stained secondary walls), oe —outer epidermis.

walls. These arise by branching from the dorsal bundle and other vascular bundles in the pedicel, or stem of the pod (Woodcock, 1934). The walls of their xylem elements are lignified. Sclerified bundle caps are not normally associated with these lateral pod bundles.

Outer epidermis and hypodermis

The outer epidermis and hypodermis have been the source of recurring texture defects in both canned and frozen green beans. On occasion they slough to a marked degree and present a ragged appearance (Kaczmarzyk *et al.*, 1963; Sistrunk, 1965; Sistrunk *et al.*, 1960; Strohmaier, 1956; Van Buren *et al.*, 1960). The basis for a sloughing of these tissues is apparent when their structure and composition are considered.

The outer epidermis of the pod at edible maturities possesses numerous stomata with extensive sub-stomatal intercellular spaces. A characteristically ridged or furrowed cuticle also is present (Fig. 9). In tangential views, the epidermal cells are prismatic in shape. Such sections through their outer cuticular areas also show that the cuticle ridges radiate from the stomata (Fig. 10). These cuticle ridges are irregularly arranged over epidermal cells more distant from the stomata.

The underlying hypodermal cells, appearing irregular in shape and size in pod cross-sections (Fig. 9), have elongate or lenticular shapes, as seen in sections tangential to the pod surface (Fig. 11). They are oriented dorsiventrally at about a 45° angle to pod length and at an opposing angle to the fiber sheath sclereids. Their cell walls thicken during edible maturity stages of pod growth and are greatly thickened when the pods are overly mature. Immediately underlying these cells are small outer parenchyma cells (compare Fig. 1 and 11).

Intercellular spaces are numerous and irregular in shape and size in this skin area, proceeding from the larger substomatal spaces to the smaller, often interconnecting spaces in the outer parenchyma. Overall, this comprises a structurally weak area at culinary maturities because of the different cellular orientations, numerous intercellular spaces, and composition of cell walls and their middle lamellae.

The walls and middle lamellae of the hypodermal and closely associated cells give strongly positive histochemical reactions for pectins and hemicelluloses. The thick secondary walls of the hypodermal cells are very hemicellulosic in test reaction. They are strongly differentiated from the primary portion of the cell wall by staining properties and appear very similar to the walls of some gelatinous fibers in which either a special condition of the cellulose or an abundance of certain hemicelluloses is believed responsible for their gelatinous, hygroscopic properties (Esau, 1965). It would appear that the twisting dehiscence of the pod valves as the ripe pod opens is controlled by the orientation and wall structure of the hypodermal and fiber sheath cells.

Freezing damage

Freezing damage is inversely related to the speed of freezing (Brown, 1967) and, in addition, the pattern of damage is related to differences in the structure and composition of the various tissues of the bean pod. In all pods frozen slowly enough to cause visible damage, there was damage to the cell walls of the smaller inner parenchyma cells closely associated with the fiber sheath (Fig. 12). Damage usually appeared as radial tears extending through several layers of these cells. With an even slower freezing rate, other cells of the inner parenchyma also were torn and radially oriented gaps were more extensive (Fig. 13).

Cell wall breakage was less extensive in the outer parenchyma, which, as previous mentioned, possess thicker walls than the inner parenchyma. Most of the tissue damage in the outer parenchyma was a separation of cells from each other along their middle lamellae, the walls remaining largely intact (Fig. 14). Cell separation also occurred to a lesser extent in the outer tissues between the hypodermis and the epidermis, or between the hypodermis and the outer parenchyma. This type of tissue sloughing is shown in Fig. 16. Both types of cell separation increased with slower freezing.

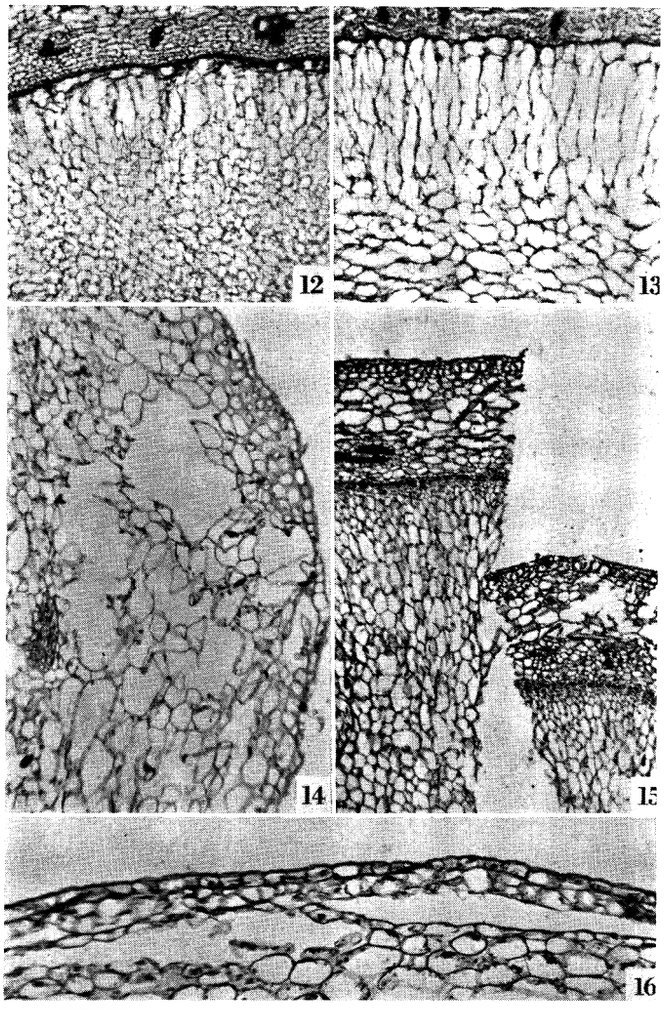

Fig. 12–16. Sections illustrating extent of freezing damage with different freezing treatments. (12) Damage to inner parenchyma near fiber sheath. (13) Damage to all portions of inner parenchyma. (15) Cracking caused by prolonged immersion in liquid nitrogen. (16) Sloughing of epidermis and hypodermis.

Freezing by immersion in liquid nitrogen prevented the development of any of thes forms of tissue damage. Prolonged immersion, however, caused cracks that either separated the pod along the dorsal and ventral sutures, or broke through all pod tissues regardless of their structure and composition (Fig. 15).

DISCUSSION

The histological origins, development and maturation of the different tissues of the green bean pod serve to explain the wide range within which specialized tissue structure and composition may variously influence textural problems encountered in processing. Similar interrelationships between special tissue conditions and texture have been encountered in other processed fruits and vegetables (Powers *et al.*, 1958; Reeve, 1964a,b, 1947, 1953, 1959, 1965; Reeve *et al.*, 1959, 1966; Sterling, 1955; and Ster-

ling *et al.*, 1961). Textural problems encountered in green beans have centered in three major areas: (1) sloughing of the skins in cooked, canned and frozen products; (2) stringiness and/or toughness due to over-maturity of fibrous tissues; and (3) loss of crispness due to the influence of over-blanching and different freezing treatments.

Skin sloughing has received some attention as a texture defect in cooked and canned products. Strohmaier (1956) and Kaczmarzyk *et al.* (1963) have illustrated that sloughing involves separation of the outer epidermis separately or in combination with its underlying hypodermis. These skin tissues are a structurally weak area with respect to processing at edible maturities. Substomatal spaces beneath the epidermis and other intercellular spaces are abundant. Other significant structural features are the marked differences in cell size, shape and cellular orientation of the elongated hypodermal cells with respect to the outer epidermis and the underlying outer parenchyma tissues.

The high pectin and other polyuronide or hemicellulosic composition of the skin area, and of the hygroscopic, apparently gelatinous nature of the hypodermal cell walls in particular, are compositional features that appear to have bearing upon susceptibility to sloughing in pods of edible maturities. At such stages of development these tissues have not as yet developed the rigidity of structure and composition characteristic of the ripened pod. Kaczmarzyk *et al.* (1963); Sistrunk (1965); and Van Buren *et al.* (1960, 1962) have related skin sloughing to pectic composition of the cell walls and found calcium treatment effective in reducing sloughing under certain conditions.

In addition to the problem of skin sloughing, it has been well demonstrated that the different tissues in the edible green pod exhibit different patterns of freezing damage according to freezing treatment (Brown, 1966; Lee *et al.*, 1946; Reeve *et al.*, 1966; Wolford *et al.*, 1965). Lee *et al.* (1946) evaluated the gross appearance, palatability and vitamin content of frozen green beans, but did not compare textural qualities or extent of structural damage in different tissue areas with respect to freezing rates.

With air-blast freezing at −10°F, both individually quick frozen and package frozen green beans have been found to be excessively damaged in both the inner and outer parenchyma tissue zones, with skin sloughing pronounced in the latter; but with complete freezing in liquid nitrogen, all tissues remained intact (Wolford *et al.*, 1965). Other experimental studies on freezing damage in frozen beans have demonstrated positive relation of even minor structural damage to texture evaluation by a sensory appraisal panel (Brown, 1967). Often this minor damage may occur only in the areas between the young bundle sheath cells and the inner and/or outer parenchyma tissues of the pod.

The cracking that occurs during prolonged liquid nitrogen freezing is frequently attributed to the change in the volume of water as it approaches the freezing point. Because it happens only after prolonged immersion in liquid nitrogen, and because the breaks do not follow cell walls or tissue differentiation, it is apparent that the bean pod is in essentially a single solid phase at the time of cracking. This form of damage probably is a result of very rapid but non-uniform contraction as the pod is cooled following the conversion of water to ice.

The relation of stringiness and fibrousness to textural quality is largely a matter of the comparative maturities of dorsal and ventral bundles with their associated sclereid caps, and of the sclereids of the fiber sheath. In addition to varietal differences in stringlessness and low fiber content at edible maturities, growing conditions are known to influence the extent of cell wall development and rates of maturation in these tissues (Kaldy, 1966; Singh *et al.*, 1966). Changes in cellulosic crystallinity, as a result of dehydration, influences texture in reconstituted vegetables (Sterling *et al.*, 1961).

In green beans such changes in cellulose are apt to be more pronounced in the fibrous tissues than in the softer parenchyma because of the smaller cells and greater amount of cell wall substance per unit volume in the former. The temperature of postharvest storage has rather pronounced effects on composition and culinary quality. Higher temperatures (*ca.* 30°C) result in gains in cellulose and water-soluble pectin, and also increase the tendency to slough (Sistrunk, 1965). Chilling temperature (5°C) reduces the shelf life and impairs culinary qualities, but the effects vary with variety (Watada *et al.*, 1966a,b).

It is obvious that there are many causes and sources of texture impairment of green beans. All, however, relate to compositional, physiological and structural characteristics of the pod tissues. A knowledge of the developmental histology of th pod should aid in distinguishing the nature and source of many of these problems of quality impairment.

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V. A. DANIEL, B. L. M. DESAI, S. VENKAT RAO, M. SWAMINATHAN and H. A. B. PARPIA
Central Food Technological Research Institute, Mysore-2, India

The Nutritive Value of Humanized Milk Food Based on Buffalo Milk and Fortified with DL-Methionine

SUMMARY—The nutritive value of infant foods (prepared from buffalo milk) containing 10%, 12.5% and 15% protein and fortified with DL-methionine has been studied in experiments with albino rats. The mean weekly growth rate of rats receiving milk food II (10% protein and 20% fat) and fortified with DL-methionine was of the same order as those obtained with milk foods containing 12.5%, 15%, 22% and 26% protein. Milk food II containing 10% protein (not fortified with DL-methionine) promoted significantly less growth than the same food fortified with DL-methionine and other milk foods containing 12.5% to 28% protein.

The protein efficiency ratio of the milk food fortified with DL-methionine (4.0) was significantly higher than that (3.3) of the unfortified milk food at 10% level of protein in the diet. The results indicate that humanized milk food from buffalo milk containing about 12.0% protein and 20% fat and fortified with DL-methionine will be suitable for feeding infants in place of full cream milk powder in developing countries where milk is in short supply. Adoption of the above formula for infant milk food manufactured in the country will help to *double* the output of infant food from the same quantity of buffalo milk without appreciable increase in cost.

INTRODUCTION

DRIED MILK FOODS used for feeding infants may be classified as follows: (1) whole (full-cream) milk powder; (2) milk powder with reduced fat content (half-cream milk powder); and (3) whole milk powder with the proportions of protein and fat altered to resemble that of dried human milk (humanized milk foods). Human milk contains about 10% protein and 26% fat on a dry basis. The milk foods commonly used for feeding infants, however, have widely varying protein (10–28%) and fat (8–26%) contents. Among the dried infant foods, humanized milk foods contain about 10–12% protein and 26% fat, similar to that of dried human milk (Meyer, 1960). Studies carried out by several workers have shown that modified cow's milk containing about 1.1 to 2.0% protein promotes nearly the same growth rate in infants as human milk (Aitken *et al.*, 1960; Mellander *et al.*, 1959; Barness *et al.*, 1957; Omans *et al.*, 1961).

It would appear from the results of these studies that a protein content of 10 to 12% in dried cow's milk formula

may be adequate for promoting normal growth of infants. It should be noted, however, that human milk protein contains larger amounts of sulphur amino acids than cow's milk proteins (Orr *et al.*, 1957) and the NPU of human milk proteins (87) is higher than that (75) of cow's milk proteins (Platt *et al.*, 1961; Venkat Rao *et al.*, 1963). It has been shown by some workers that the protein efficiency ratio (PER) of cow's milk proteins can be increased by supplementation with the limiting amino acid methionine (Henry *et al.*, 1953). The objectives of the present study were to assess by experiments with albino rats (1) the effect of supplementation with methionine on the protein efficiency ratio of buffalo milk proteins and (2) the nutritive value of dried milk foods from buffalo milk containing 10, 12.5 and 15.0% proteins and 20% fat and fortified with DL-methionine.

MATERIALS AND METHODS

THE SPRAY-DRIED WHOLE-MILK POWDER used in this study was manufactured from buffalo milk by the Kaira District Co-operative Milk Producer's Union, Anand. Malto-dextrin was supplied by the Raptakos Brett, Bombay. Buffalo ghee (clarified butter fat) and cane sugar were obtained from the local market.

Infant milk foods

Infant milk foods containing varying levels of protein were prepared by blending in a mechanical mixer different quantities of whole milk powder, ghee (clarified butter fat), cane sugar and malto-dextrin as indicated in Table 1. The different foods were fortified with calcium phosphate, ferric ammonium citrate and vitamin premix to maintain the same content of essential minerals and vitamins in all the foods. The chemical composition of the different foods as determined by AOAC methods (1955) is given in Table 2. A part of the infant foods containing 10, 12.5 and 15% protein was fortified with DL-methionine to raise the sulphur amino acid content to 4.4g/16gN, i.e., to the same level as in human milk proteins. The essential amino acid composition of the proteins of buffalo milk determined by microbiological methods (Barton-Wright, 1952) as compared with those of cow's and human milks is given in Table 3.

Animal experiments

Experiments were conducted on albino rats to determine (1) the protein efficiency ratio of dried buffalo milk and buffalo milk casein fortified with DL-methionine and (2) the overall growth promoting value of infant foods

Table 1. Composition of different infant milk foods based on buffalo milk.

Milk food	Full-fat milk powder (g)	Ghee (clarified butter fat) (g)	Tri-calcium phosphate (g)	Ferric-ammonium citrate (mg)	Cane sugar (g)	Malto-dextrin (g)	Vitamin premix (g)[1]
Milk food I—corresponding to full cream milk powder (cow's)	100	.....	.....	.....	.....	.....	.....
Milk food II—humanized milk food (10% protein)	35.7	10	1.8	35	27.5	23.2	1.8
Milk food III—humanized milk food (12.5% protein)	44.6	7.5	1.5	35	24.4	20.5	1.5
Milk food IV—(containing 15% protein)	53.6	5.0	1.2	35	21.4	17.6	1.2
Milk food V—(corresponding to ISI standards)	78.6	.....	0.6	35	9.4	10.8	0.6

[1] 3 g of vitamin premix contained the same amounts of B-vitamins present in 100 g of whole milk powder.

Table 2. Chemical composition of different milk foods (values per 100 g).

Nutrients	Milk foods				
	I	II	III	IV	V
Moisture (g)	2.6	2.4	2.5	2.5	2.6
Fat (g)	26.1	20.1	20.2	20.3	20.2
Protein (N × 6.25 g)	28.2	10.2	12.7	15.1	22.2
Ash (g)	6.1	4.1	4.3	4.5	5.4
Carbohydrates (by diff) (g)	37.0	63.2	60.3	57.6	49.6
Calcium (g)	1.22	1.18	1.20	1.17	1.24
Phosphorus (g)	0.85	0.83	0.91	0.94	0.96
Iron (mg)	6.2	6.3	6.4	6.1	6.2
Thiamine (mg)	0.32	0.35	0.36	0.34	0.35
Riboflavin (mg)	0.72	0.73	0.70	0.72	0.75
Niacin (mg)	0.82	0.85	0.91	0.87	0.88
Vitamin C (mg)	14.2	14.3	14.8	14.3	14.5
Vitamin A (I.U.)	1560	1610	1510	1620	1560
Vitamin D (I.U.)	400	400	400	400	400
Calorific value (KCal)	496	475	484	474	469
Protein (g/100KCal)	5.7	2.2	2.6	3.2	4.8

Table 3. Essential amino acid composition of the proteins of buffalo milk, cow's milk and human milk (g/16gN).

Amino acid	Buffalo	Cow[1]	Human[1]
Isoleucine	6.6	6.4	6.4
Leucine	9.3	9.9	8.9
Lysine	7.6	7.8	6.3
Phenylalanine	5.1	4.9	4.6
Tyrosine	5.0	5.1	5.5
Cystine	1.1	0.9	2.1
Methionine	2.5	2.4	2.2
Total sulphur amino acids	3.6	3.3	4.3
Threonine	4.5	4.6	4.6
Tryptophan	1.4	1.4	1.6
Valine	6.8	6.9	6.6

[1] Ref: FAO (1965) Protein Requirements Rep. No. 37.

(with and without added DL-methionine) based on buffalo milk.

Protein efficiency ratio (PER)

The PER of the proteins of dried buffalo milk and buffalo milk casein (with and without added DL-methionine) was determined by the method of Osborne *et al.* (1919). The diets contained 10% protein (N × 6.25). The composition of the diets was similar to that described by Shurpalekar *et al.* (1964). Male albino rats (21 days old and 7 rats per group) were allotted to different groups by a randomized block design. The animals were housed in individual cages with raised wire-mesh bottoms. The diets were mixed with twice the amount of water and fed to the animals. Records of the daily food intake and weekly increase in body weight of the animals were maintained. The experiment lasted for a period of 4 weeks. The results are given in Table 4.

Overall nutritive value of infant milk foods

The overall nutritive value of the different infant milk foods was determined by the rat growth method described by Shurpalekar *et al.* (1963). Young male albino rats (28 days old and 8 rats per group) were allotted to different groups by a randomized block design. The animals were housed in individual cages with raised metal-screen bottoms. The milk foods were mixed with twice the weight of water and fed to the animals. Records of daily food intake and weekly gain in body weight of animals were maintained.

Table 4. Effect of supplementation of buffalo milk and buffalo milk casein with DL-methionine on the protein efficiency ratio. (Mean values of 7 male rats per group; experiment duration, 4 weeks).

Diets	Protein intake (g/4 weeks)	Gain in weight (g/day)	PER (at 10% protein level)
Buffalo milk	24.75	2.93	3.31
Buffalo milk + DL-methionine	30.06	4.30	4.00
Casein (from buffalo milk)	22.10	2.37	3.26
Casein + DL-methionine	24.45	3.43	3.93
Standard error of the mean		± 0.13 (18 df)	± 0.08 (18 df)

At the end of the experimental period of 8 weeks, hemoglobin and red blood cell counts were determined in blood drawn from the tail veins according to Korula *et al.* (1960). The animals were anesthetized with ethyl ether and the liver was removed for determination of fat and histological structure. Fat was determined according to Korula *et al.* (1960) and the histological structure according to Tasker *et al.* (1962). The results are given in Table 5.

RESULTS

Protein efficiency ratio (Table 4)

Fortified buffalo milk with DL-methionine brought about a marked increase in the PER from 3.2 to 4.0. A similar improvement in the PER of buffalo milk casein was observed as a result of fortification.

Growth rate

No significant differences were observed in the mean weekly growth rates of rats fed on infant foods I (28% protein), infant food II (10% protein) fortified with DL-methionine, infant food III (12.5% protein), infant food IV (15% protein) and infant food V (22.0% protein). Infant food II containing 10% protein (not fortified with DL-methionine), however, promoted significantly less growth than the other infant foods.

Blood composition

There were no significant differences in the hemoglobin and RBC count of the blood of rats fed on the different infant milk foods.

Table 5. Growth rate, RBC and hemoglobin of young albino rats fed on infant foods of varying protein levels with or without DL-methionine supplementation. (Mean values for 8 male rats per group; experiment duration, 8 weeks).

Diets	Protein %	Initial weight (g)	Gain in weight (g/week)	Blood composition: Hemoglobin (g/100 ml blood)	Blood composition: RBC (10^6/cu mm blood)	Composition of liver: Fresh weight (g)	Composition of liver: Moisture %	Composition of liver: Fat %
1. Whole milk powder (IF I)	28.2	55.9	20.44	13.9	4.78	7.6	70.7	3.46
2. Infant food II (IF)	10.2	55.6	16.45	13.6	4.58	6.8	68.9	5.61
3. Infant food II + DL-meth.	10.3	55.4	20.89	13.8	4.65	7.6	69.8	3.75
4. Infant food III	12.7	55.8	22.75	13.7	4.62	8.1	70.2	3.40
5. Infant food III + DL-meth.	12.8	55.8	22.75	13.7	4.68	8.0	70.0	3.33
6. Infant food IV	15.1	55.1	22.58	14.0	4.82	8.0	70.9	3.24
7. Infant food IV + DL-meth.	15.2	55.5	23.83	14.1	4.90	8.1	69.6	3.24
8. Infant food V	22.2	55.5	22.17	14.2	4.96	8.2	69.8	3.10
Standard error of the mean (49 df)			± 0.89	± 0.2	±0.08		± 0.4	±0.07

Liver composition

The mean liver fat contents of rats fed on infant food II fortified with DL-methionine and of those fed on infant foods I, III, IV and V were within normal limits. The mean fat content of livers of rats fed on infant food II containing 10% protein, however, was significantly higher than that of livers of rats fed on other infant foods. It is of interest to note that fortification of infant food II with DL-methionine reduced the liver fat content.

Histological structure of liver

The livers of animals receiving infant food II containing 10% protein showed a mild generalized fatty infiltration that was corrected by fortification with DL-methionine. The livers of animals fed on infant foods I, III, IV and V and infant food fortified with DL-methionine were quite normal.

DISCUSSION

THE RESULTS OBTAINED show that an infant food containing 10 to 12.5% protein and fortified with DL-methionine promotes very good growth in young albino rats comparing well with that produced by full-fat milk powder (28% protein and 26% fat) and a proprietory infant food containing about 22% protein and 18% fat according to I.S.I. standards (I.S.I., 1960). Data regarding the relative quantities and cost of buffalo milk and other materials required for the production of 1 kg of infant food (I.S.I. standard) and infant food (humanized) containing 12.0% protein and 20% fat are given in Table 6. For the production of 1 kg of infant food containing 22% protein and 18% fat about 5.5 kg of full-fat buffalo milk and 0.25 kg of cane sugar will be required. About 200 g butter fat will be obtained as a by-product.

On the other hand, 3 kg of full-fat buffalo milk (7.5% fat, 4.0 protein and 17.0% total solids) mixed with 0.5 kg of malto-dextrin will yield 1 kg of infant milk food containing 12.0% protein and 20% fat. Such a food can be fortified with vitamins and minerals and DL-methionine at a low cost. *Adoption of the above formula for infant milk food will help to double the output of infant food from the same quantity of buffalo milk without appreciable increase in cost.* In view of the shortage in milk production in India, there is an urgent need for the adoption of a revised standard of 12.0% protein and 20% fat fortified with DL-methionine for the infant foods manufactured in the country.

Table 6. Relative cost of raw materials for the production of infant food containing 12% and 22% protein from buffalo milk.

Items	Infant food (22% protein and 18% fat-ISI standard)	Infant food (12% protein and 20% fat (humanized)
Raw materials:		
Buffalo milk (kg)	5.5	3.0
Cane sugar or Malto-dextrin (kg)	0.25	0.5
DL-methionine (g)	.....	1.5
Final products:		
Butterfat (g)	200	.....
Infant food (kg)	1	1
Cost:		
Raw materials (Rupees)	6.0	4.1
Butterfat (by product) (Rupees)	2.0	.....
Cost of raw materials per kg infant food (Rupees)	4.0	4.1

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ELWOOD F. REBER, MARK H. BERT, ELIZABETH M. RUST and ERIC KUO
Department of Nutrition and Food, University of Massachusetts, Amherst, Massachusetts 01002

Biological Evaluation of Protein Quality of Radiation-Pasteurized Haddock, Flounder and Crab

SUMMARY—The effects of radiation sub-sterilization or heat-treatment on the protein quality of haddock, crab and flounder were evaluated by determining the protein efficiency ratio (PER). The PER value of haddock radiated at either level was statistically equal to that of non-radiated haddock. The PER value of crab radiated at either level was statistically equal to non-radiated crab in one replication and significantly higher than non-radiated crab in another replication. The PER value of flounder radiated at the low level was significantly less than that of non-radiated flounder, whereas the PER value of flounder radiated at the high level was statistically equal to that of non-radiated flounder. Low PER values were obtained for heat-treated marine products and these values were significantly lower than those for all other marine product treatments investigated. The protein quality of haddock, crab and flounder was not significantly changed as a result of radiation pasteurization.

INTRODUCTION

STUDIES HAVE BEEN SUPPORTED by the Office of the U.S. Surgeon General and by the Quartermaster Corps of the U.S. Army on foods radiated at sterilization levels of 3 or 6 megarads. The results of these studies have shown that radiation-sterilized foods are wholesome and contain no toxic factors. The loss of nutrients is of the same magnitude as that encountered in thermal sterilization. It is recognized that the loss of nutrients caused by radiation treatment might be decreased if a lower dose of radiation (1 megarad or less) adequate to pasteurize foods is used (Reber *et al.*, 1966).

Various results have been reported by workers (Borgstrom *et al.*, 1962; Rand et al., 1960) using different methods of determining the effects of heat-treatment on the nutritive value of fish products. In most cases, chemical analyses showed no appreciable decrease in the essential amino acid content of processed (canned, frozen or boiled) fish products, whereas biological assays showed a slight decrease in the essential amino acid content of heat-treated fish products. Dehydration caused no detectable impairment of haddock protein digestibility. Sun- and air-dried fish and fresh fish promoted the same growth in rats (Borgstrom *et al.*, 1962). According to De Groot (1963), freeze-dehydration caused negligible change in the protein quality of cooked fish meal.

This is a report on the biological determination of the protein qualities of radiation-pasteurized, heat-treated and non-radiated haddock, flounder and crab.

MATERIALS AND METHODS

THE FOLLOWING MARINE PRODUCTS were used in this work: haddock *(Melanogrammus aeglefinus)*, flounder *(Limanda ferruginea)*, and crab *(Cancer magister)*.

The haddock and flounder came from the Atlantic Ocean and the crab from the Pacific. The supply of each of these marine products, packed in No. 10 cans, was divided into 4 equal portions. The first portion was heat-treated for 4⅓ hr at 116°C and then stored at ambient temperature until shipped for freeze-dehydration. The second portion was frozen immediately and stored in a freezer at 0–2°C until shipped for freeze-dehydration; this portion served as the non-radiated control. The third portion was radiated at a low pasteurization level. The fourth portion was radiated at a high pasteurization level. These 2 portions were stored for 30 days at 0–2°C.

The marine products were radiated with Co-60 gamma rays. The radiation doses used for the low or high pasteurization levels for flounder were 0.3 and 0.6 megarads, and for haddock and crab were 0.2 and 0.4 megarads.

The thawed marine products, including their juices, were freeze-dehydrated as follows: Each product was loaded onto oven trays to a depth of 1–4 cm, frozen at −30°C, and then quickly transferred to a dehydration chamber. The chamber was evacuated immediately, and dehydration was continued at a pressure not exceeding 1.5 mm of mercury. When this pressure was obtained, the temperature of the water entering the platens was raised to 38°C and maintained at that temperature for 22 hr. The marine products became essentially dry. Then the platen temperature was lowered to room temperature, and maintained there for 72 hr. Air was admitted to the dehydration chamber until atmospheric pressure was reached. The marine products were removed from the chamber and placed in No. 2.5 cans. The cans and their contents were twice evacuated and flushed with nitrogen to lower the oxygen content of the head space gas to less than 2%. The cans were sealed under nitrogen.

The protein quality of the freeze-dehydrated marine products was determined according to the official method of biological evaluation of protein quality (Horwitz, 1960). The results of the proximate analyses of the freeze-dehydrated products are shown in Table 1. There was no fiber present in any of the samples.

The experimental diets contained 10% protein (N × 6.25), and were stored at −15°C. Aliquots of the refrigerated diets were removed from storage immediately prior to offering them to male albino rats of the Holtzman strain, which had been weaned at not less than 21 days and not more than 28 days. The rats were assembled into groups of 10 each, replicated twice for each treatment.

In the assay of each marine product, a group of rats fed Animal Nutrition Research Council (ANRC) reference casein served as control groups. Throughout the 28-day

Table 1. The proximate analyses (%) of the freeze-dehydrated marine products.

	Protein	Fat	Moisture	Ash
Haddock				
Heat-treated, non-radiated	91.0	1.70	2.33	5.32
Raw, non-radiated	89.8	1.77	3.12	5.83
Raw, radiated (0.2 megarad)	90.0	1.55	3.48	5.86
Raw, radiated (0.4 megarad)	89.6	1.45	2.87	6.30
Crab				
Heat-treated, non-radiated	78.3	3.58	2.53	13.4
Raw, non-radiated	75.0	2.48	3.64	15.7
Raw, radiated (0.2 megarad)	78.5	2.52	3.87	13.8
Raw, radiated (0.4 megarad)	78.5	3.01	3.48	13.3
Flounder				
Heat-treated, non-radiated	92.6	3.82	1.86	7.30
Raw, non-radiated	84.5	3.04	3.02	6.82
Raw, radiated (0.3 megarad)	92.1	3.23	2.54	7.56
Raw, radiated (0.6 megarad)	90.3	3.80	3.03	7.46

assay period, each rat was kept in an individual cage in an air-conditioned room and was provided with appropriate assay diet and water *ad libitum*. The body weight of each rat was recorded at the start of the assay period and at 7-day intervals. Quantitative measurements of the diets offered to the rats and the spilled and refused diet were made at intervals of 7 days. Total weight gain and protein intake during the 28-day period were calculated for each rat and, in turn, the averages for each group.

Protein efficiency ratio (PER), namely the body weight gain divided by the protein intake, for each rat and the average for each group were calculated. The ratio of the PER for each marine product to the PER for the ANRC casein was calculated. The protein quality of the sample is reported as the above ratio × 100.

An analysis of variance of the PER values, rather than the protein quality values, was carried out; thus, the information and degrees of freedom contained in the PER values for ANRC casein were utilized, and the efficiency of the experiment was maximized. The statistical significance of differences between treatment means for PER values was applied to the corresponding differences between treatment means for protein quality values.

RESULTS AND DISCUSSION

ACCORDING TO the least significant differences (LSD) shown under Table 2, the protein quality values of 113, 119 and 118 for non-radiated haddock, haddock radiated at the 0.2 megarad level, and haddock radiated at the 0.4 megarad level, respectively, are statistically equal. These results are in agreement with the findings of Brooke *et al.* (1966) in which the amino acid content of haddock fillets radiated at 0.15, 0.25 or 2.5 megarads was not changed as compared to non-radiated controls.

Table 2. The mean PER and protein quality values for haddock.

	Casein	Non-radiated	0.2 megarad	0.4 megarad	Heat-treated
Rep. 1	2.97	3.39	3.82	3.41	2.50
Rep. 2	3.00	3.30	3.27	3.61	2.61
Mean[1]	2.98	3.38	3.54	3.51	2.55
Protein quality	100	113	119	118	86

[1] The variance for treatment × replication and for replication are homogeneous to the error variance, but the variance for treatments is significantly ($P < 0.01$) large. Standard error of treatment mean PER value = 0.090. Least significant difference (LSD) between treatment mean PER values: 0.272 ($P = 0.05$) and 0.359 ($P = 0.01$) for two-tailed t; 0.228 ($P = 0.05$) and 0.324 ($P = 0.01$) for one-tailed t.

Table 3. The PER[1] and protein quality values for crab.

	Casein	Non-radiated	0.2 megarads	0.4 megarads	Heat-treated
Rep. 1	2.97	2.83	3.66	3.29	0.95
Rep. 2	3.00	3.70	3.78	3.32	1.96
Rep. 1	100	95	123	111	32
Rep. 2	100	123	126	111	65

[1] The variance for treatment × replication interaction, for treatments, and for replications are significantly ($P < 0.01$) large. Standard error of group mean PER value = 0.161. LSD between group mean PER values: 0.452 ($P = 0.05$) and 0.599 ($P = 0.01$) for two-tailed t; 0.378 ($P = 0.05$) and 0.546 ($P = 0.01$) for one-tailed t.

Table 3 shows the group mean PER values obtained for crab and the corresponding protein quality values. The ANRC reference casein control in the haddock assay also served as the control in the crab assay. Because the variance for the treatment × replication interaction is significantly ($P<0.01$) large, only group mean PER values within the same replication should be compared. According to the LSD values shown under Table 3, in replication 1 the protein qualities of crab radiated at the 0.2 or 0.4 megarad level are statistically equal, and are significantly ($P<0.05$) higher than the protein quality of non-radiated crab. In replication 2, the protein quality of crab radiated at the 0.2 megarad level is statistically equal to that of non-radiated crab, and is just significantly ($P = 0.05$) higher than that of crab radiated at the 0.4 megarad level.

These results correspond to previous findings where the superior PER values of fish as compared to casein were attributed to the abundance of lysine existing in fish tissues (Morrison *et al.*, 1960; Borgstrom *et al.*, 1962). Radiation at either level did not significantly change the protein quality of haddock or crab except in replication 1 where the PER value for crab radiated at either level was significantly higher that that of non-radiated crab.

Table 4 shows the group mean PER values obtained for flounder and the protein quality values corresponding to the treatment mean PER values. According to the LSD values shown under Table 4 the protein quality of flounder radiated at the 0.3 megarad level is significantly ($P<0.01$)

Table 4. The mean PER and protein quality values for flounder.

	Casein	Non-radiated	0.3 megarads	0.6 megarads	Heat-treated
Rep. 1	3.02	3.93	3.62	3.73	2.51
Rep. 2	3.32	4.07	3.74	4.10	2.78
Mean[1]	3.17	4.00	3.68	3.92	2.64
Mean protein quality	100	126	116	123	83

[1] The variance for the treatment × replication is homogeneous to the error variance, and the variances for treatments and for replications are significantly ($P < 0.01$) large. Standard error of treatment mean PER value = 0.065. LSD between treatment PER values: 0.170 ($P = 0.05$) and 0.224 ($P = 0.01$) for two-tailed t; 0.142 ($P = 0.05$) and 0.202 ($P = 0.01$) for one-tailed t.

lower than that of non-radiated flounder; the protein quality of flounder radiated at the 0.6 megarad level was statistically equal to that of non-radiated flounder.

The heat-treated portion of each marine product had a protein quality significantly ($P<0.01$) less than the non-radiated portion. The protein quality of 86 for heat-treated haddock was significantly ($P < 0.01$) lower than the value of 113 for non-radiated haddock. The low PER values for the heat-treated samples provides a striking contrast between them and raw or radiated samples. The growth of rats fed the heat-treated products was remarkably poor. Obviously, the proteins were damaged or the digestibility of the proteins was decreased by heating the product in No. 10 cans for 4⅓ hr at 116°C. There are several workers who state that carefully-controlled processing does not alter the nutritive value of fish; also that raw shellfish protein was more digestible than the cooked form (Borgstrom *et al.*, 1962).

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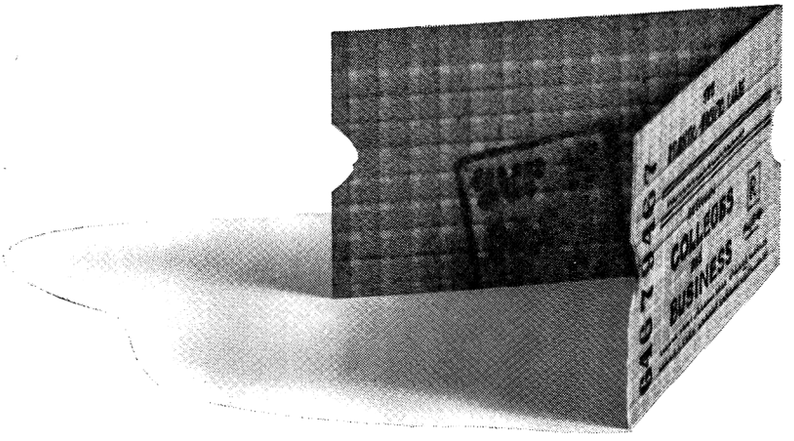

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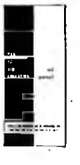

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